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FOREWORD

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5. Introduction

A major goal of cancer research has been to identify tumor antigens which are qualitatively or quantitatively different from normal cells (1). The presence of such antigens could be detected by monoclonal antibodies that would form the basis of diagnostic and prognostic tests. In addition, the antibodies could be used to selectively kill tumor cells either directly via their effector function (2) or by attaching cytotoxic molecules to the antibody (3, 4).

Despite the demonstration of antigens which are overexpressed on tumor cells, antibodies have been used with limited success for diagnosis and treatment of solid tumors, (reviewed in ref. (5, 6)). Their utility has been hampered by the paucity of tumor specific antibodies, immunogenicity, low affinity, and poor tumor penetration. For this project, we proposed using a novel technology, termed phage display, to produce a new generation of antibodies which would overcome the limitations of previously produced anti-tumor antibodies (ref. 7-11). The antibodies would bind breast cancer antigens with high affinity, be entirely human in sequence, and would penetrate tumors better than IgG.

5.1. Purpose of the present work and methods of approach

For this work, we proposed to isolate and characterize a large assortment of high affinity human and murine antibody fragments that bound to specific breast cancer antigens and to normal antigens that are overexpressed on cancer cells. Antibodies isolated using phage display would be used for early sensitive diagnosis of node-negative breast cancer patients, for immunotherapy prior to growth of large tumor mass, and as adjuvant therapy for minimal residual disease. Human antibodies were to be isolated from a very large and diverse phage antibody library of >6,700,000,000 different members (12). Murine antibodies would be isolated from libraries constructed from the B-lymphocytes of mice immunized with breast tumor cell lines. Antibodies that recognize antigens which were overexpressed or unique to breast carcinomas would be isolated by selection on breast tumor cell lines and characterized with respect to affinity and specificity.

The proposed technical objectives in the statement of work were:

- Task 1: Create phage antibody libraries from mice immunized with malignant breast tissue and with the tumor cell lines MDA MB231, ZR-75-1 and SKBR3 (months 1-18).
- Immunize mice with appropriate cell line or tissue.
 - Prepare mRNA, amplify V_H and V_L genes, create scFv gene repertoires.
 - Construct phage antibody libraries.
- Task 2: Create subtractive phage antibody libraries from mice immunized with malignant breast tissue and with the tumor cell lines MDA MB231, ZR-75-1 and SKBR3 (months 1-18).
- Immunize mice with appropriate normal cell line or tissue and deplete repertoire with Cytoxan.
 - Immunize mice with the appropriate tumor cell line or tissue.
 - Prepare mRNA, amplify V_H and V_L genes, create scFv gene repertoires.
 - Construct phage antibody libraries.
- Task 3: Isolate and characterize scFv antibody fragments which bind novel breast tumor antigens by selecting phage antibody libraries on malignant breast tissue, paraffin embedded malignant breast tissue and the tumor cell lines MDA MB231, ZR-75-1 and SKBR3. (months 12-30).
- Determine optimal conditions for selecting cell surface binding phage antibodies using C6.5 ErbB2 binding phage antibody, an irrelevant hapten binding phage antibody and ErbB2 expressing SK-OV-3 cells.
 - Determine optimal conditions for selecting internalizing phage antibodies using C6.5 ErbB2 binding phage antibody and SKBR3 cells.

- c. Select a 6.7×10^9 member human phage antibody library on malignant breast tissue, paraffin embedded malignant breast tissue and the tumor cell lines MDA MB231, ZR-75-1 and SKBR3.
- d. Select phage antibody libraries constructed in tasks 1 and 2 on malignant breast tissue, paraffin embedded malignant breast tissue and the tumor cell lines MDA MB231, ZR-75-1 and SKBR3.
- e. Determine scFv specificity on a panel of cell lines and tissues.

Task 4: Determine the antigens recognized by tumor specific scFv using Western blotting, immunoaffinity purification, and protein sequencing. (months 24-36).

- a. Create scFv affinity columns for antigen immunopurification.
- b. Identify tumor specific antigens by Western blotting, immunoaffinity purification and protein sequencing.
- c. Create subtractive tumor cell line phage cDNA library.
- d. Select tumor cell phage cDNA library on purified monoclonal scFv which recognize tumor specific antigens.

6. Body of report

When we proposed and submitted this project in early 1997, it was unclear as to the likelihood of obtaining tumor cell specific antibodies by selecting phage antibody libraries directly on tumor cell lines. While our group was the first to report the successful selection of cell binding antibodies from phage libraries by direct selection on erythrocytes (13), reports of subsequent successful cell selections have been interspersed with reports of failures. Thus we proposed using both large non-immune phage antibody libraries and libraries constructed from mice immunized with tumor cell lines. The advantage of large non-immune phage libraries is that they can be constructed from human variable region genes and thus yield human antibodies, ideal for use as therapeutics. The advantage of using murine libraries is that at least in theory the libraries can be enriched for antibodies which bind the immunizing cell line.

Since the construction of phage antibody libraries is a difficult and time consuming task, we focused during the first year of funding on determining the factors affecting the successful selection of cell binding phage antibodies using a model system (Task 3a and 3b). Once the optimal method of selection was determined, proof of concept was demonstrated using a non-immune human phage antibody library (Task 3c). This work followed on work begun in the latter year of funding from DAMD17-94-J-4433 and continued through the first year of this project.

6.1. Identification of factors influencing successful selection of phage antibodies directly on tumor cells

To determine the factors affecting the successful selection of phage antibodies on tumor cells, we employed a model system using a previously isolated human phage antibody, C6.5. C6.5 binds the tumor antigen ErbB2 with a $K_d = 1.6 \times 10^{-8}$ M and is a stable monomeric single chain Fv antibody fragment (scFv) in solution with no tendency to spontaneously dimerize or aggregate (14). To determine the impact of affinity on successful cell selections, we studied a phage antibody (C6ML3-9) which differs from C6.5 by 3 amino acids (15). C6ML3-9 binds the same epitope as C6.5 but with a 16 fold lower K_d (1.0×10^{-9} M) (14). To examine the affect of avidity on efficiency of cell selections, we also studied the dimeric C6.5 diabody (16). Diabodies are scFv dimers where each chain consists of heavy (V_H) and light (V_L) chain variable domains connected using a peptide linker which is too short to permit pairing between domains on the same chain. Consequently, pairing occurs between complementary domains of two different chains, creating a stable noncovalent dimer with two binding sites (17). The C6.5 diabody was constructed by shortening the peptide linker between the Ig V_H and V_L domains from 15 to 5

amino acids and binds ErbB2 on SKBR3 cells bivalently with a K_d approximately 40 fold lower than C6.5 (4.0×10^{-10} M) (16).

For determination of the efficiency of cell selections, the C6.5 and C6ML3-9 scFv and C6.5 diabody genes were subcloned for expression as pIII fusions in the phagemid pHEN-1 (10). This should yield phagemid predominantly expressing a single scFv or diabody-pIII fusion after rescue with helper phage (see figure 2 in Becerril et al., appendix 1). Diabody phagemid display a bivalent antibody fragment resulting from intermolecular pairing of one scFv-pIII fusion molecule and one native scFv molecule. The C6.5 scFv gene was also subcloned into the phage vector fd-Sfi/Not. This results in phage with 3 to 5 copies each of scFv-pIII fusion protein (see figure 2 in Becerril et al., appendix 1). The human breast cancer cell line SKBR3 was used as a target cell line for cell binding. Its surface ErbB2 density is approximately 1.0×10^6 per cell.

First, binding of the different phage antibodies to ErbB2 expressing SKBR3 cells was determined by titrating phage eluted from the cell surface. After washing with phosphate buffered saline (PBS), surface bound phage were eluted from the cell surface by sequential washes with pH 2.8 glycine buffer. The titer of anti-ErbB2 eluted phage was compared to the titer of an irrelevant anti-botulinum antibody (Tables 1 and 2, Becerril et al., appendix 1). After elution with glycine, enrichment ratios (specific phage titer/irrelevant phage titer) were only 2 fold for the C6.5 phage and only 8.9 fold for the 1 nM K_D C6ML3-9 phage antibody. These meager enrichment ratios could be increased slightly by sequential elutions with pH 2.8 glycine, such that after three elutions the enrichment ratios increased to 2.7 and 11.4 respectively (Table 2, Becerril et al., appendix 1). These enrichment ratios are probably too low to allow successful selection of rare binders from a library. The efficiency of selection could be increased significantly to 8 fold after 1 glycine elution and 20 fold for the third glycine elution for the multivalent C6.5 phage. This ratio is more compatible with successful selection. Enrichment ratios could be increased further, by lysing the cells after three glycine washes, leading to recovery of 'intracellular' phage (Table 2, Becerril et al., appendix 1). We assume the phage recovered after cell lysis were 'intracellular', since the titer increased 7 to 40 fold from the third glycine wash. Titers were highest for antibody formats capable of dimerizing the receptor (diabody and phage), with enrichment ratios of 30 to 146 fold (Table 2, Becerril et al., appendix 1). Such enrichment ratios are very likely to lead to successful selection from a library.

To confirm that phage were endocytosed, immunofluorescent microscopy was performed using the phage antibodies after stripping the cell surface with pH 2.8 glycine (figure 3, Becerril et al., appendix 1). The results confirmed that phage bearing an antibody to an internalizing cell surface receptor can be endocytosed and that the endocytosis was most efficient for dimeric (diabody) or multimeric (phage) formats capable of crosslinking the receptor (figure 3, Becerril et al., appendix 1).

To reduce the background of non-specific phage recovery, we studied the effect of trypsinizing the cells prior to cell lysis. This should remove phage trapped in the extracellular matrix. Trypsinization also dissociates the cells from the cell culture flask, permitting transfer to a new vessel and elimination of any phage bound to the cell culture flask. We found that trypsinization resulted in a 60 fold reduction in non-specific binding with only a minor reduction in the amount of specific phage recovery (figure 4, Becerril et al., appendix 1).

Only very large phage antibody libraries containing more than 5.0×10^9 members are capable of generating panels of high affinity antibodies to all antigens (12, 18). Since phage can only be concentrated to approximately 10^{13} cfu/ml, a typical phage preparation from a large library will only contain 10^4 copies of each member. Thus selection of libraries for cell binding or endocytosis could only work if phage can be recovered when applied to cells at titers as low as 10^4 . We therefore determined the recovery of infectious phage from within SKBR3 cells as a function of the phage titer applied. Phage recovery increased with increasing phage titer for all

phage studied (figure 6, Becerril et al., appendix 1)). For monovalently displayed antibodies, phagemid could not be recovered from within the cell at input titers less than 3.0×10^5 (C6.5 scFv) to 3.0×10^6 (C6ML3-9 scFv). This threshold decreased for bivalent and multivalent display (3.0×10^4 for C6.5 diabody phagemid and C6.5 scFv phage).

Similar results for cell binding were observed when using SK-OV-3 cells, however titers of endocytosed phage were significantly lower compared to SKBR3 cells, presumably due to alternate splicing of the ErbB2 receptor which results in decreased endocytic capacity.

The results demonstrate that phage displaying an anti-receptor antibody can be specifically endocytosed by receptor expressing cells and can be recovered from the cytosol in infectious form. The results demonstrate the feasibility of directly selecting cell binding and internalizing antibodies from large non-immune phage libraries and identify the factors that will lead to successful selections. When monovalent scFv antibody fragments were displayed monovalently in a phagemid system, recovery of cell surface bound phage was only 2 to 9 fold above background. This ratio is too low to allow successful selection. Even when phage was recovered after endocytosis. Enrichment ratios were only 3.5 to 18 fold above background. Display of bivalent diabody or multivalent display of scFv in a phage vector increased recovery of internalized phage to 30 to 146 fold above background. This result is consistent with our studies of native monomeric C6.5 scFv and dimeric C6.5 diabody as well as studies of other monoclonal anti-ErbB2 antibodies where dimeric IgG but not monomeric Fab dimerize and activate the receptor and undergo endocytosis (19, 20). In fact it is likely that endocytosis of C6.5 and C6ML3-9 scFv phagemids reflect the small percentage of phage displaying two or more scFv. The importance of valency in mediating either high avidity binding or receptor crosslinking and subsequent endocytosis is confirmed by the only other report demonstrating specific phage endocytosis. Phage displaying approximately 300 copies of a high affinity Arg-Gly-Asp integrin binding peptide on pVIII were efficiently endocytosed by mammalian cells (21). Recovery of phage after endocytosis also increases the specificity of cell selections compared to recovery of phage from the cell surface. Thus enrichment ratios for specific vs non-specific surface binding range from 2 to 20 fold. These values are comparable to the approximately 10 fold enrichment reported by others for a single round of cell surface selection (22, 23). In contrast our enrichment ratios for specific vs non-specific endocytosis range from 3.5 to 146 fold.

Significance: Based on these results, selection of cell binding or internalizing antibodies from phage antibody libraries would be most successful with either homodimeric diabodies in a phagemid vector or multivalent scFv using a phage vector. Multivalent libraries would present the antibody fragment in the form most likely to crosslink receptor and undergo endocytosis. Antibodies from such libraries would need to be bivalent to mediate endocytosis. Alternatively, monomeric receptor ligands can activate receptors and undergo endocytosis, either by causing a conformational change in the receptor favoring the dimeric form or by simultaneously binding two receptors. Monomeric scFv that bound receptor in a similar manner could also be endocytosed. Thus selection of libraries of monovalent scFv in a phagemid vector could result in the selection of ligand mimetics that activate receptors and are endocytosed as monomers. Such scFv could be especially useful for the construction of fusion molecules for the delivery of drugs, toxins or DNA into the cytoplasm. Since antibodies which mediate receptor internalization can cause receptor down regulation and growth inhibition (20, 23-26), selection for endocytosable antibodies may also identify antibodies which directly inhibit or modulate cell growth.

6.2 Selection and characterization of cell binding and internalizing antibodies from a phage antibody library

The results described in section 6.1 encouraged us to attempt selection of a non-immune phagemid antibody library to identify new phage antibodies that were bound and were internalized into SKBR3 cells (Task 3c). A selection strategy illustrated in Nielsen & Marks,

figure 4, appendix 2 was utilized. After 3 rounds of selection, 40% of randomly picked clones bound SKBR3 cells and of these, 50% bound ErbB2 by ELISA (Table 2, Poul et. al., appendix 3). This is not surprising, given that SKBR3 cells express very high levels of ErbB2. DNA fingerprinting indicated that 2 unique antibodies (F5 and C1) binding ErbB2 were obtained. These stained ErbB2 expressing cell lines comparably to other ErbB2 antibodies and in proportion to the cell lines known level of ErbB2 expression (Table 3, Poul et. al., appendix 3). Many additional antibodies were obtained that did not bind ErbB2 and that preferentially bound tumor cell lines but not normal cell lines (Table 3, Poul et. al., appendix 3). One of these antibodies which stained a number of tumor cell lines (H7) was studied further. The H7 gene was subcloned into a secretion vector and native hexahistidine tagged scFv was purified and used to immunoprecipitate the antigen it recognized from an SKBR3 cell lysate. After excision from a gel and protein sequencing, the antigen recognized by H7 was determined to be the transferrin receptor. F5 stained ErbB2 in Western blot (figure 2, Poul et. al., appendix 3), and both F5 and H7 could immunoprecipitate their respective antigens from SKBR3 cell lysates (figure 2, Poul et. al., appendix 3). As either phage antibodies or native monomeric scFv antibody fragment, both F5 and H7 were efficiently endocytosed by SKBR3 cells (figure 3 and 4, Poul et. al., appendix 3). In the case of H7 (but not F5) endocytosis served as a surrogate marker for growth inhibition, with H7 exhibiting dose dependent inhibition of the growth of SKBR3 cells (figure 5, Poul et. al., appendix 3). H7 competed with holotransferrin for binding to the transferrin receptor, explaining probably both the mechanism of growth inhibition and the mechanism by which it was able to induce receptor mediated endocytosis of the transferrin receptor (figure 6, Poul et. al., appendix 3).

The results demonstrate that tumor specific phage antibodies can be directly selected from phage libraries by panning on tumor cell lines and recovering phage which have triggered receptor mediated endocytosis from within the cytosol. Such antibodies are efficiently endocytosed by the target cell line, both as phage antibodies and as native scFv antibody fragments. As such, these antibodies are likely to be ideal for delivery of drugs or genes into the cytosol for therapeutic application. In some instances (as with H7), endocytosis can be used as a surrogate marker for direct desirable biologic effects exhibited by the antibody, in this case growth inhibition.

6.3 Generation of a non-immune phage antibody library in a true phage vector

Our results above indicate that cell surface selections are most efficient (give the highest enrichment ratios) when the scFv antibody fragment is displayed in multiple copies on the surface of bacteriophage. This occurs when the phage antibody library is constructed in a true phage vector containing all of the phage genome. To date, all non-immune and most immune phage antibody libraries have been constructed in phagemid vectors. The remainder of the phage genes and proteins are provided by infecting *E. coli* harboring the phagemid antibody with a helper phage. Since the helper phage provides wild type pIII, the majority of phage antibodies have only a single copy of scFv-pIII fusion protein, with the remaining 4 copies of pIII being wild type. Libraries to date have been constructed in phagemid vectors for two reasons: 1) the transformation efficiencies are much higher, making it easier to construct large libraries; and 2) the concern that multivalent display may lead to selection of lower affinity phage antibodies due to avidity.

Our data indicates that even with very high affinity (1 nM) antigen binding, cell surface selection results in very low enrichment ratios, even when binding an internalizing receptor and recovering phage from within the cell. Thus construction of immune phagemid libraries (as proposed in tasks 1 and 2) did not make sense. Rather we chose to construct true phage libraries. Given the technical difficulties in generating large phage libraries from cDNA, to validate the utility of phage libraries for generating antibodies binding cell surface antigens, we elected to construct a true phage antibody library by subcloning the scFv gene repertoire from our existing phagemid library (ref. 12) into a phage vector into which we have engineered compatible cloning sites for the scFv gene repertoire (fd-TET/Sfi-Not). This allows preparation

of large quantities of phagemid vector harboring the scFv gene repertoire from which the scFv gene repertoire can be excised as Sfi-Not restriction enzyme fragments. fd-TET/Sfi-Not vector DNA was prepared by digestion with the same two restriction enzymes and the scFv gene repertoire ligated into vector DNA. After multiple transformations (> 50) a library containing 5.0×10^8 transformants was obtained. PCR screening of 20 randomly selected colonies indicated that 100% had a scFv sized insert and fingerprinting indicated that the library was diverse. In the coming year, we will characterize this library with respect to its ability to generate antigen specific antibodies and compare the number of antibodies and their binding constants with those obtained from our phagemid library.

6.4 Generation of a high throughput assay for cell binding and endocytosis

One factor limiting our ability to identify recombinant phage antibodies which bind and internalize into tumor cells is a high throughput assay for cell binding and endocytosis. While we have used cell ELISA, it has a high background and only reports cell binding, not endocytosis. As we have been working with liposomes, a means occurred to us to generate a high throughput assay for endocytosis. Our scFv can easily be engineered to have a C-terminal hexahistidine tag. This can be achieved by batch subcloning the output scFv gene repertoire after each round of selection. We have been able to construct liposomes containing a fluorescent reporter dye and having on their surface Ni-NTA which has been inserted into the lipid coat of the liposome. We hypothesize that such liposomes should be able to chelate the hexahistidine tagged scFv (directly from the bacterial supernatant without the need for purification). If the scFv binds an internalizing epitope, the fluorescent liposome will enter the cell. ScFv and liposomes remaining on the cell surface can easily be removed by washing with EDTA. Cells are then lysed and if the scFv binds an internalizing epitope, a fluorescent signal will occur. Using Ni-NTA liposomes and the internalizing and non-internalizing scFv we have generated to date, we will validate this assay and determine its sensitivity.

7. Key research accomplishments

- Identification of optimal phage antibody format for selection of phage antibodies on tumor cells
- Demonstration that phage antibodies binding cell surface receptors can trigger receptor mediated endocytosis
- Identification of optimal phage antibody format for selection of internalizing phage antibodies on tumor cells
- Successful selection of tumor specific phage antibodies from a non-immune phage antibody library
- Construction of a large non-immune phage antibody library in a true phage vector

8. Reportable outcomes

- 8.1 Becerril, B., Poul, M.-A., and Marks, J.D. Toward selection of internalizing phage antibodies. *Biochem Biophys Res. Comm.* 255: 386-393, 1999.
- 8.2 Nielsen, U.B. and Marks, J.D. Internalizing antibodies and targeted cancer therapy: direct selection from phage libraries. *Pharmaceutical Sciences and Trends Today.* 3: 282-291, 2000.
- 8.3 Poul, M.-A., Becerril, B., Nielsen, U.B., Morisson, P., and Marks, J.D. Selection of tumor-specific internalizing human antibodies from phage libraries. *J. Mol. Biol.* 301: 1149-1161, 2000.

9. Conclusions

- 9.1. We have identified conditions which allow successful selection of cell binding and internalizing phage antibodies by panning phage libraries directly on cells (Tasks 3a and 3b).

- 9.2 Using a model system, we show that cell surface selection and selection for endocytosis is more efficient when the phage antibody is multivalent
- 9.3 In the same model system, cell selections are more efficient when phage are recovered after endocytosis than when recovered from the cell surface.
- 9.4 We have demonstrated successful selection of tumor specific antibodies by panning a non-immune phage antibody library on the SKBR3 tumor cell line (Task 3c).
- 9.5 During the coming year, we will characterize additional internalizing phage antibodies resulting from this selection with respect to specificity and antigen recognized. We will attempt to identify antigens which are tumor specific and heretofore unknown.
- 9.6. We are constructing and characterizing true multivalent phage libraries to lead to more efficient selection of cell binding and internalizing antibodies.
- 9.7 We are developing a high throughput assay to identify cell binding and internalizing antibodies after selection.

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11. Appendices

Toward Selection of Internalizing Antibodies from Phage Libraries

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Antibodies which bind cell surface receptors in a manner whereby they are endocytosed are useful molecules for the delivery of drugs, toxins, or DNA into the cytosol of mammalian cells for therapeutic applications. Traditionally, internalizing antibodies have been identified by screening hybridomas. For this work, we studied a human scFv (C6.5) which binds ErbB2 to determine the feasibility of directly selecting internalizing antibodies from phage libraries and to identify the most efficient display format. Using wild-type C6.5 scFv displayed monovalently on a phagemid, we demonstrate that anti-ErbB2 phage antibodies can undergo receptor-mediated endocytosis. Using affinity mutants and dimeric diabodies of C6.5 displayed as either single copies on a phagemid or multiple copies on phage, we define the role of affinity, valency, and display format on phage endocytosis and identify the factors that lead to the greatest enrichment for internalization. Phage displaying bivalent diabodies or multiple copies of scFv were more efficiently endocytosed than phage displaying monomeric scFv and recovery of infectious phage was increased by preincubation of cells with chloroquine. Measurement of phage recovery from within the cytosol as a function of applied phage titer indicates that it is possible to select for endocytosable antibodies, even at the low concentrations that would exist for a single phage antibody member in a library of 10^9 . © 1999 Academic Press

Key Words: endocytosis; ErbB2; gene therapy; phage display; single-chain Fv antibodies; targeted gene delivery.

Growth factor receptors are frequently overexpressed in human carcinomas and other diseases and thus have been utilized for the development of targeted therapeutics. The *HER2/neu* gene, for example, is amplified in several types of human adenocarcinomas,

especially in tumors of the breast and the ovary (1) leading to the overexpression of the corresponding growth factor receptor ErbB2. Targeting of ErbB2 overexpressing cells has been accomplished primarily using anti-ErbB2 antibodies in different formats, including conjugation to liposomes containing chemotherapeutics (2), fusion to DNA carrier proteins delivering a toxic gene (3), and direct fusion to a toxin (4). For many of these targeted approaches, it is necessary to deliver the effector molecule across the cell membrane and into the cytosol. This can be accomplished by taking advantage of normal growth factor receptor biology; growth factor binding causes receptor activation via homo- or heterodimerization, either directly for bivalent ligand or by causing a conformational change in the receptor for monovalent ligand, and receptor mediated endocytosis (5). Antibodies can mimic this process, stimulate endocytosis, become internalized and deliver their payload into the cytosol. In general, this requires a bivalent antibody capable of mediating receptor dimerization (6, 7). In addition, the efficiency with which antibodies mediate internalization differs significantly depending on the epitope recognized (7, 8). Thus for some applications, such as liposomal targeting, only antibodies which bind specific epitopes are rapidly internalized and yield a functional targeting vehicle.

Currently, antibodies which mediate internalization are identified by screening hybridomas. Alternatively, it might be possible to directly select internalizing antibodies from large non-immune phage libraries (9, 10) by recovering infectious phage particles from within cells after receptor mediated endocytosis, as reported for peptide phage libraries (11, 12). Unlike the multivalently displayed peptide phage libraries, however, phage antibody libraries typically display monomeric single chain Fv (scFv) or Fab antibody fragments fused to pIII as single copies on the phage surface using a phagemid system (9, 10). We hypothesized that such monovalent display was unlikely to lead to efficient receptor crosslinking and phage internalization. To de-

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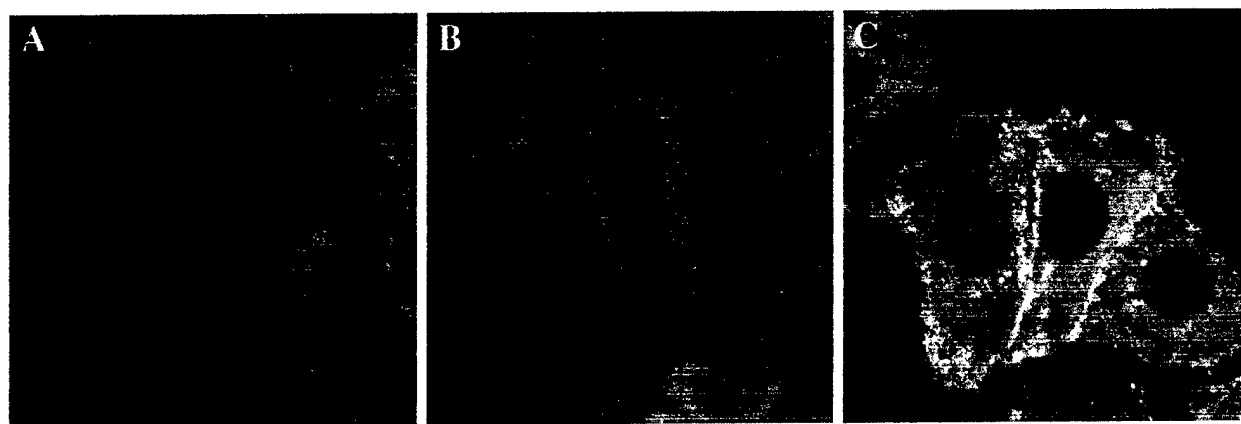


FIG. 1. Internalization of soluble C6.5 scFv and diabody. SKBR3 cells grown on cover slips were incubated with (A) anti-botulinum scFv (B) C6.5 scFv or (C) C6.5 diabody for 2 h at 37°C. Coverslips were washed with PBS and stripping buffer, cells were fixed and permeabilized and intracellular antibodies were detected by confocal microscopy using the anti-myc antibody 9E10, anti-mouse biotinylated antibody and streptavidin Texas-Red.

termine the feasibility of selecting internalizing antibodies and to identify the most efficient display format, we studied a human scFv (C6.5) which binds ErbB2 (13). Using wild type C6.5 scFv, we demonstrate that anti-ErbB2 phage antibodies can undergo receptor mediated endocytosis. Using affinity mutants and dimeric diabodies of C6.5 displayed as either single or multiple copies on the phage surface, we define the role of affinity, valency, and display format on phage endocytosis and identify the factors that lead to the greatest enrichment for internalization. The results indicate that it is possible to select for endocytosable antibodies, even at the low concentrations that would exist for a single phage antibody member in a library of 10^9 members.

MATERIAL AND METHODS

Cells. The SKBR3 breast tumor cell line was obtained from ATCC and grown in RPMI media supplemented with 10% FCS (Hyclone) in 5% CO₂ at 37°C.

Antibodies and antibody phage preparations. The C6.5 scFv phage vector was constructed by subcloning the C6.5 gene as a *SfiI/NotI* fragment from scFv C6.5 pHEN1 (13) into the phage vector *fd/SfiI/NotI* (a gift of Andrew Griffiths, MRC Cambridge, UK). The C6.5 diabody phagemid vector was constructed by subcloning the C6.5 diabody gene (14) as a *NcoI/NotI* fragment into pHEN1 (15). The anti-botulinum scFv phagemid (clone 3D12) (16) C6.5 scFv phagemid (13) and scFv C6ML3-9 scFv phagemid (17) in pHEN1 have been previously described. Phage were prepared (18) from the appropriate vectors and titered on *E. coli* TG1 as previously described (9) using ampicillin (100 µg/ml) resistance for titration of constructs in pHEN1 and tetracycline (50 µg/ml) for titration of constructs in *fd*. Soluble C6.5 scFv, C6.5 diabody and anti-botulinum scFv were expressed from the vector pUC119mycHis (13) and purified by immobilized metal affinity chromatography as described elsewhere (13).

Detection of internalized native antibody fragments and phage antibodies. SKBR3 cells were grown on coverslips in 6-well culture

plates (Falcon) to 50% of confluency. Culture medium was renewed 2 h prior to the addition of 5×10^{11} cfu/ml of phage preparation (the phage preparation representing a maximum of 1/10 of the culture medium volume) or 20 µg/ml of purified scFv or diabody in phosphate buffered saline, pH 7.4 (PBS). After 2 h of incubation at 37°C, the wells were quickly washed 6 times with ice cold PBS and 3 times for 10 min each with 4 mL of stripping buffer (50 mM glycine pH 2.8, 0.5 M NaCl, 2M urea, 2% polyvinylpyrrolidone) at RT. After 2 additional PBS washes, the cells were fixed in 4% paraformaldehyde (10 min at RT), washed with PBS, permeabilized with acetone at -20°C (30 s) and washed again with PBS. The coverslips were saturated with PBS-1% BSA (20 min. at RT). Phage particles were detected with biotinylated anti-M13 immunoglobulins (5 Prime-3 Prime, Inc, diluted 300 times) (45 min at RT) and Texas red-conjugated streptavidin (Amersham, diluted 300 times) (20 min. at RT). Soluble scFv and diabodies containing a C-terminal myc peptide tag were detected with the mouse mAb 9E10 (Santa Cruz Biotech, diluted 100 times) (45 min. at RT), anti-mouse biotinylated immunoglobulins (Amersham, diluted 100 times) and Texas red-conjugated streptavidin. Optical confocal sections were taken using a Bio-Rad MRC 1024 scanning laser confocal microscope. Alternatively, slides were analyzed with a Zeiss Axioskop UV fluorescent microscope.

Recovery and titration of cell surface bound or internalized phage. Subconfluent SKBR3 cells were grown in 6-well plates. Culture medium was renewed 2 h prior to the experiment. Cells were incubated for varying times with different concentrations of phage preparation at 37°C (specific details for each experiment are provided in the table or figure legends). Following PBS and stripping buffer washes, performed exactly as described above for detection of internalized native antibody fragments and phage antibodies, the cells were washed again twice with PBS and lysed with 1 mL of 100 mM triethylamine (TEA). The stripping buffer washes and the TEA lysate were neutralized with 1/2 volume of Tris-HCl 1M, pH 7.4. For some experiments (see figure legends for specifics), cells were trypsinized after the three stripping buffer washes, collected in a 15-ml Falcon tube, washed twice with PBS and then lysed with TEA. In experiments performed in the presence of chloroquine, SKBR3 cells were preincubated for 2 h in the presence of complete medium containing 50 µM chloroquine prior to the addition of phage. Corresponding control samples in the absence of chloroquine were prepared at the same time. For all experiments, phage were titered on *E. coli* TG1 as described above.

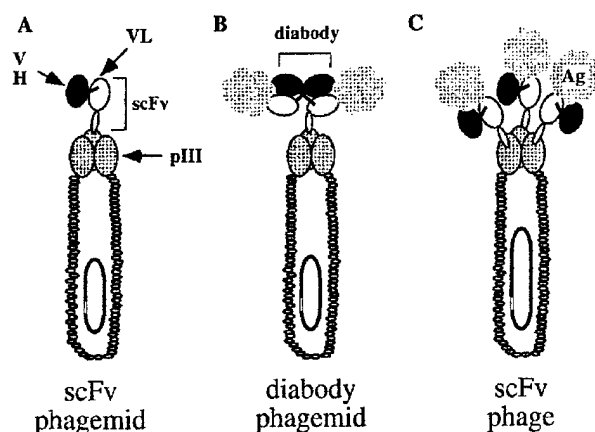


FIG. 2. Antibody phage display. Cartoon of phage displaying (A) a single scFv (B) a single diabody or (C) multiple scFv. scFv, single chain Fv antibody fragment; V_H , Ig heavy chain variable domain; V_L , Ig light chain variable domain; pIII, phage minor coat protein pIII; Ag, antigen bound by scFv.

RESULTS

1. The Model System Utilized to Study Phage Antibody Internalization

The human anti-ErbB2 scFv C6.5 was obtained by selecting a human scFv phage antibody library on recombinant ErbB2 extracellular domain (13). C6.5 scFv binds ErbB2 with a $K_d = 1.6 \times 10^{-8}$ M and is a stable monomeric scFv in solution with no tendency to spontaneously dimerize or aggregate (13). To determine the impact of affinity on internalization, we studied a scFv (C6ML3-9) which differs from C6.5 by 3 amino acids (17). C6ML3-9 scFv is also a stable monomer in solution and binds the same epitope as C6.5 scFv but with a 16-fold lower K_d (1.0×10^{-9} M) (17, 19). Since receptor homodimerization appears to typically be requisite for antibody internalization we also studied the dimeric C6.5 diabody (14). Diabodies are scFv dimers where each chain consists of heavy (V_H) and light (V_L) chain variable domains connected using a peptide

linker which is too short to permit pairing between domains on the same chain. Consequently, pairing occurs between complementary domains of two different chains, creating a stable noncovalent dimer with two binding sites (20). The C6.5 diabody was constructed by shortening the peptide linker between the Ig V_H and V_L domains from 15 to 5 amino acids and binds ErbB2 on SKBR3 cells bivalently with a K_d approximately 40-fold lower than C6.5 (4.0×10^{-10} M) (14).

Native C6.5 scFv and C6.5 diabody was expressed and purified from *E. coli* and analyzed for endocytosis into ErbB2 expressing SKBR3 breast tumor cells by immunofluorescent confocal microscopy. As expected, monomeric C6.5 scFv is not significantly internalized whereas the dimeric C6.5 diabody can be detected in the cytoplasm of all cells visualized (Fig. 1).

For subsequent experiments, the C6.5 and C6ML3-9 scFv and C6.5 diabody genes were subcloned for expression as pIII fusions in the phagemid pHEN-1 (15). This should yield phagemid predominantly expressing a single scFv or diabody-pIII fusion after rescue with helper phage (21) (Figs. 2A and 2B). Diabody phagemid display a bivalent antibody fragment resulting from intermolecular pairing of one scFv-pIII fusion molecule and one native scFv molecule (Fig. 2B). The C6.5 scFv gene was also subcloned into the phage vector fd-Sfi/Not. This results in phage with 3 to 5 copies each of scFv-pIII fusion protein (Fig. 2C). The human breast cancer cell line SKBR3 was used as a target cell line for endocytosis. Its surface ErbB2 density is approximately 1.0×10^6 per cell (22).

2. C6.5 Phagemids Are Endocytosed by Human Cells

C6.5 scFv phagemids were incubated for 2 h with SKBR3 cells grown on coverslips at 37°C to allow active internalization. Cells were extensively washed with PBS to remove non specific binding and then washed an additional three times with high salt and low pH (stripping) buffer to remove phage specifically bound to cell surface receptors. Internalized phagemid were de-

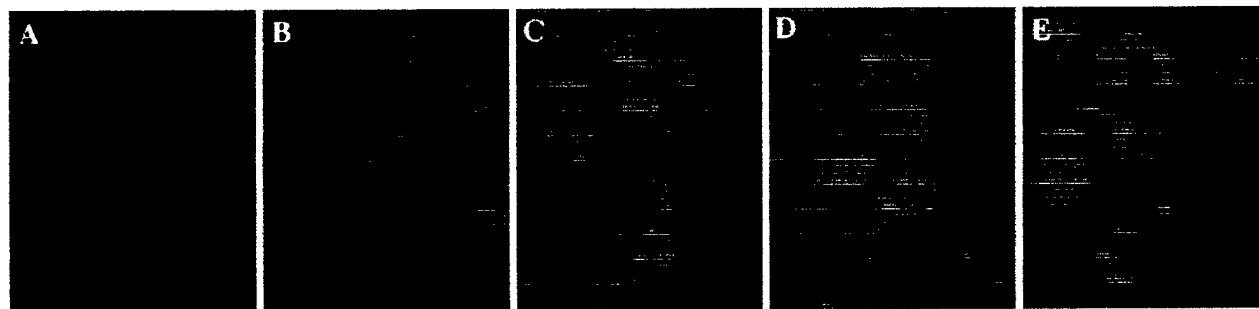


FIG. 3. Internalization of C6.5 phage derivatives. SKBR3 cells grown on coverslips were incubated with 5.0×10^{11} cfu/ml (A) anti-botulinum phagemid (B) scFv C6.5 phagemid (C) C6ML3-9 phagemid (D) C6.5 diabody phagemid or (E) C6.5 phage for 2 h at 37°C. The cells were treated as described in the legend to Fig. 1 and intracellular phage were detected with a fluorescent microscope using biotinylated anti-M13 antiserum and Texas-Red streptavidin.

TABLE 1
Titration of Membrane-Bound and Intracellular Phage

Phage antibody	Cell surface phage titer ($\times 10^{-5}$)			Intracellular phage titer ($\times 10^{-5}$)
	1st wash	2nd wash	3rd wash	
Anti-botulinum phagemid	280	36	2.8	15
C6.5 scFv phagemid	600	96	7.6	52
C6ML3-9 scFv phagemid	2500	140	32	270
C6-5 diabody phagemid	1800	120	13	450
C6.5 scFv phage	2300	620	56	2200

Note. 3.0×10^{11} cfu of monovalent C6.5 scFv phagemid, 16-fold higher affinity monovalent C6ML3-9 scFv phagemid, bivalent C6.5 diabody phagemid, or multivalent C6.5 fd phage were incubated with subconfluent SKBR3 cells for 2 h at 37°C. Cells were washed 6 times with PBS, 3 times with stripping buffer, and then lysed to recover intracellular phage. The various fractions were neutralized and the phage titered. The total number of cfu of each fraction is reported. Nonspecific anti-botulinum phagemid were used to determine nonspecific recovery.

tected with a biotinylated M13 antiserum recognizing the major coat phage protein pVIII. An anti-botulinum toxin phagemid was used as a negative control. Staining was analyzed by using immunofluorescent microscopy (Fig. 3). Approximately 1% of the cells incubated with C6.5 scFv phagemid showed a strong intracellular staining consistent with endosomal localization (Fig. 3B) while no staining was observed for anti-botulinum phagemid (Fig. 3A). Furthermore, no staining was seen if the incubation was performed for 2 h at 4°C instead of 37°C (data not shown). Staining performed after the PBS washes but before washing with stripping buffer showed membrane staining of all the cells, indicating that multiple washes with stripping buffer is necessary to remove surface bound phagemids. The results also indicate that only a fraction of the cell bound phage are endocytosed.

3. Increased Affinity and Bivalency Lead to Increased Phage Endocytosis

We compared the internalization of C6.5 scFv, C6ML3-9 scFv and C6.5 diabody phagemid and C6.5 scFv phage using immunofluorescence. Both C6ML3-9 scFv and C6.5 diabody phagemid as well as C6.5 scFv phage yielded increased intensity of immunofluorescence observed at the cell surface compared to C6.5 scFv phagemid. For C6ML3-9 scFv phagemid, approximately 10% of the cells showed intracellular fluorescence after 2 h of incubation (Fig. 3C). This value increased to approximately 30% of cells for the dimeric C6.5 diabody phagemid (Fig. 3D) and 100% of cells for multivalent C6.5 scFv phage (Fig. 3E).

4. Infectious Phage Can be Recovered from within the Cell and Their Titer Correlates with the Level of Uptake Observed Using Immunofluorescence

To determine if infectious phage antibody particles could be recovered from within the cell, we incubated

approximately 5.0×10^5 SKBR-3 cells for 2 h at 37°C with 3.0×10^{11} cfu of the different phagemid or phage. Six PBS washes were used to remove non-specifically bound phage and specifically bound phage were removed from the cell surface by three consecutive washes with stripping buffer (washes I, II, and III respectively, Table 1). The cells were then lysed with 1 mL of a 100 mM triethylamine solution (TEA) (representing the intracellular phage). The three stripping washes and the cell lysate were neutralized and their phage titer was determined by infection of *E. coli* TG1. The titers of phage recovery are reported in Table 1.

Considerable background binding was observed in the first stripping wash for the anti-botulinum phage even after 6 PBS washes (2.8×10^7 cfu, Table 1). This value likely represents phage non-specifically bound to the cell surface as well as phage trapped in the extracellular matrix. The amount of surface bound phage increased only 2.1-fold above this background for C6.5 scFv phagemid (Tables 1 and 2). With increasing affinity and avidity of the displayed C6.5 antibody fragment, the titer of cell surface bound phagemid or phage increased (Table 1). The titer of phage in the consecutive stripping washes decreased approximately 10-fold with each wash. These additional stripping washes led to a minor increase in the titer of specific phage eluted compared to the background binding of the anti-botulinum phage (2.7-fold for C6.5 scFv phagemid to 20-fold for C6.5 scFv phage, Table 2). The only exception was the titer of the C6.5 diabody phagemid, where the ratio actually decreased from 6.4- to 4.6-fold. This is likely due to the fact that in the diabody the V_H and V_L domains that comprise a single binding site are not covalently attached to each other via the peptide linker. This increases the likelihood that a stringent eluent (like glycine) could dissociate V_H from V_L and abrogate binding to antigen.

Three stripping washes were required to ensure that the titer of phage recovered after cell lysis was greater

TABLE 2
Specific Enrichment of Anti-ErbB2 Phage Compared to Anti-Botulinum Phage

Phage antibody	Anti-ErbB2/anti-botulinum phage titer ratio ^a			Intracellular/cell surface phage ratio ^b
	Cell surface (1st wash)	Cell surface (3rd wash)	Intracellular	
C6.5 scFv phagemid	2.14	2.7	3.5	6.8
C6ML3-9 scFv phagemid	8.9	11.4	18	8.4
C6.5 diabody phagemid	6.4	4.6	30	35
C6.5 scFv phage	8.2	20	146	39

^a The titers of anti-ErbB2 phage are divided by the titers of the anti-botulinum phage (Table 1) to derive an enrichment ratio for specific vs nonspecific binding or internalization.

^b The titer of intracellular phage is divided by the titer of cell surface bound phage (Table 1) to derive the ratio of internalized phage vs surface bound phage.

than the titer in the last stripping wash (Table 1). We presumed that after three stripping washes, the majority of the phage eluted represented infectious particles from within the cell rather than from the cell surface. In fact, since the cell lysate titer observed with non-specific anti-botulinum phage was considerable (1.5×10^6) and greater than observed in the last stripping wash, it is likely that many phage remain trapped within the extracellular matrix and relatively inaccessible to the stripping buffer washes. Some anti-botulinum phage might also be non-specifically endocytosed by cells, but this is likely to be a small amount given the immunofluorescence results (Fig. 3). The titer of phage in the TEA fraction increased with increasing affinity and avidity of C6.5, with the highest titers observed for the dimeric C6.5 diabody phagemid and the multivalent C6.5 scFv phage (Table 1). The values represent a 30-fold (C6.5 diabody phagemid) and 146-fold (C6.5 scFv phage) increase in titer compared to the anti-botulinum phage (Table 1). We have presumed that the increase in the phage titer in the cell lysate compared to the last stripping wash is due to endocytosed phage. In fact, some of these phage could have come from the cell surface or intracellular matrix. While this could be true for a fraction of the phage from the cell lysate, the immunofluorescence results indicate that at least some of the phage are endocytosed. One indicator of the relative fraction of endocytosed phage for the different C6.5 molecules is to compare the amount of phage remaining on the cell surface prior to cell lysis (last stripping wash) with the amount recovered after cell lysis. This ratio shows only a minor increase for monovalent C6.5 scFv or C6ML3-9 scFv phagemid (6.8- and 8.4-fold, respectively) compared to anti-botulinum phagemid (5.4) (Table 2). In contrast the ratios for dimeric C6.5 diabody phagemid and multivalent C6.5 scFv phage increase to a greater extent (35 and 39, respectively) compared to anti-botulinum phagemid.

5. Increasing the Enrichment Ratios of Specifically Endocytosed Phage

The results above indicate that phage antibodies can undergo receptor mediated endocytosis and remain infectious in a cell lysate. Selection of internalized phages from a phage library requires the optimization of the method to increase enrichment of specifically internalized phages over non-internalized phage. Two parameters can be improved: (i) reduction of the recovery of non-specific or non-internalized phage and (ii) preservation of the infectivity of internalized phage. To examine these parameters, we studied wild-type C6.5 scFv phagemid. We chose this molecule because it was clearly endocytosed based on confocal microscopy, yet was the molecule undergoing the least degree of specific endocytosis. C6.5 scFv phagemid also represents the most commonly utilized format for display of non-immune phage antibody libraries (single copy pIII in a phagemid vector) and has an affinity (16 nM) more typical of K_d 's of scFv from such libraries than the affinity matured C6ML3-9 scFv (10, 23).

a. Reducing the background of non-internalized phage. To reduce the background of nonspecific phage recovery, we studied the effect of trypsinizing the cells prior to TEA lysis. This should remove phage trapped in the extracellular matrix. Trypsinization also dissociates the cells from the cell culture flask, permitting transfer to a new vessel and elimination of any phage bound to the cell culture flask. For these experiments, C6.5 scFv phagemid (5.0×10^8 ampicillin resistant cfu) were mixed with a 1000-fold excess of wild type fd phage (5.0×10^{11} tetracycline-resistant cfu). After incubation of phagemid with SKBR-3 cells for 2 h at 37°C, cells were washed with PBS and three times with stripping buffer. Cells were then directly lysed with TEA or treated with trypsin, washed twice with PBS and then lysed with TEA. Phagemid in the first stripping wash and the cell lysate were titered by infection of *E. coli* TG1 and plated on ampicillin and tetracycline

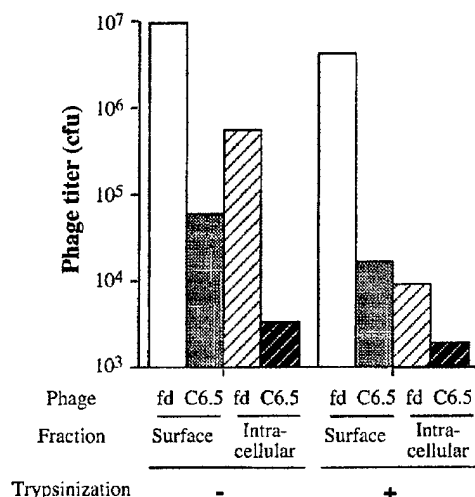


FIG. 4. Effect of trypsinization on the enrichment of antigen specific phage. A mixture of fd phage (5.0×10^{11} cfu) and C6.5 scFv phagemid (5.0×10^8 cfu) was incubated with SKBR3 cells for 2 h at 37°C. Washes were performed either as described in Table 1 (-) or cells were trypsinized prior to cell lysis (+). Phage present in the first stripping buffer wash (cell surface phage) and the cell lysate (intracellular phage) were titrated in the presence of ampicillin (C6.5 phagemid) or tetracycline (fd phage).

plates. The titer of fd phage and C6.5 scFv phagemid recovered from the cell surface was comparable for the two experimental groups (Fig. 4). The ratio of fd phage/C6.5 scFv phagemid in the cell surface fractions (160/1 and 250/1) yields a 4- to 6-fold enrichment achieved by specific cell surface binding from the initial 1000-fold ratio. Without trypsinization, the ratio of fd phage/C6.5 scFv phagemid in the cell lysate increases only 6.1-fold; in contrast, the ratio increases 209-fold with trypsinization (Fig. 4). This results from a 60-fold reduction in nonspecific binding with only a minor reduction in the amount of specific phage recovery (Fig. 4).

b. Improving the recovery of infectious internalized phage. To increase the recovery of infectious internalized phage, we studied whether prevention of lysosomal acidification through the use of chloroquine would protect endocytosed phages from endosomal degradation (12). SKBR3 cells were incubated with chloroquine and either C6.5 scFv phagemid or anti-botulinum phagemid. Cell lysates were titrated at various time points to determine the number of intracellular phagemid. C6.5 scFv phagemid were present at the 20-min time point and the amount of phagemid was comparable with or without the addition of chloroquine. At later time points, approximately twice as much infectious phagemid was recovered with the use of chloroquine. In contrast, much lower amounts of anti-botulinum phage were present and chloroquine had no effect on the titer, suggesting that the phagemid result from non-specific surface binding rather than non-specific en-

docytosis into endosomes. Overall, the results indicate that prevention of lysosomal acidification increases the amount of infectious phage recovered for incubations longer than 20 min (Fig. 5).

6. Recovery of Internalized Phage at Low Phage Concentrations

Only very large phage antibody libraries containing more than 5.0×10^9 members are capable of generating panels of high affinity antibodies to all antigens (10, 23, 24). Since phage can only be concentrated to approximately 10^{13} cfu/ml, a typical phage preparation from a large library will only contain 10^4 copies of each member. Thus selection of libraries for endocytosis could only work if phage can be recovered when applied to cells at titers as low as 10^4 . We therefore determined the recovery of infectious phage from within SKBR3 cells as a function of the phage titer applied. SKBR3 cells were incubated with C6.5 scFv, C6ML3-9 scFv or C6.5 diabody phagemids or C6.5 scFv phage for 2 h at 37°C. Cells were washed three times with stripping buffer, trypsinized and washed twice with PBS. Cells were lysed and intracellular phage titrated on *E. coli* TG1. Phage recovery increased with increasing phage titer for all phage studied (Fig. 6). For monovalently displayed antibodies, phagemid could not be recovered from within the cell at input titers less than 3.0×10^5 (C6.5 scFv) to 3.0×10^6 (C6ML3-9 scFv). This threshold decreased for bivalent and multivalent display (3.0×10^4 for C6.5 diabody phagemid and C6.5 scFv phage).

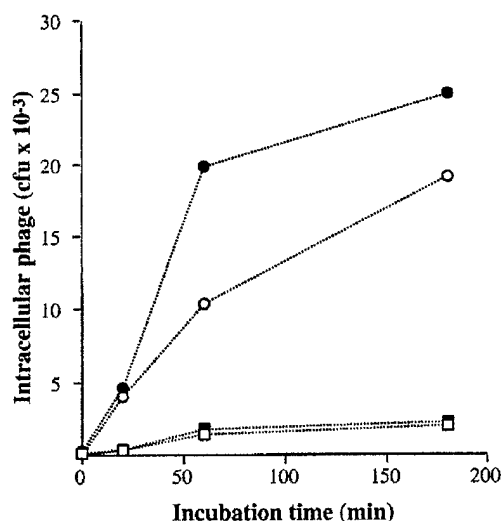


FIG. 5. Effect of incubation time and chloroquine on the recovery of antigen specific phage. SKBR3 cells were incubated in the presence (■, ●) or absence (□, ○) of chloroquine (50 μ M) for 2 h prior to the addition of anti-botulinum phagemid (□, ■) or C6.5 scFv phagemid (○, ●) (1.5×10^9 cfu/ml). Cell samples were taken at 0 min, 20 min, 1 h, or 3 h after phage addition, washed as described in the legend to Fig. 4 including the trypsinization step and intracellular phages titrated.

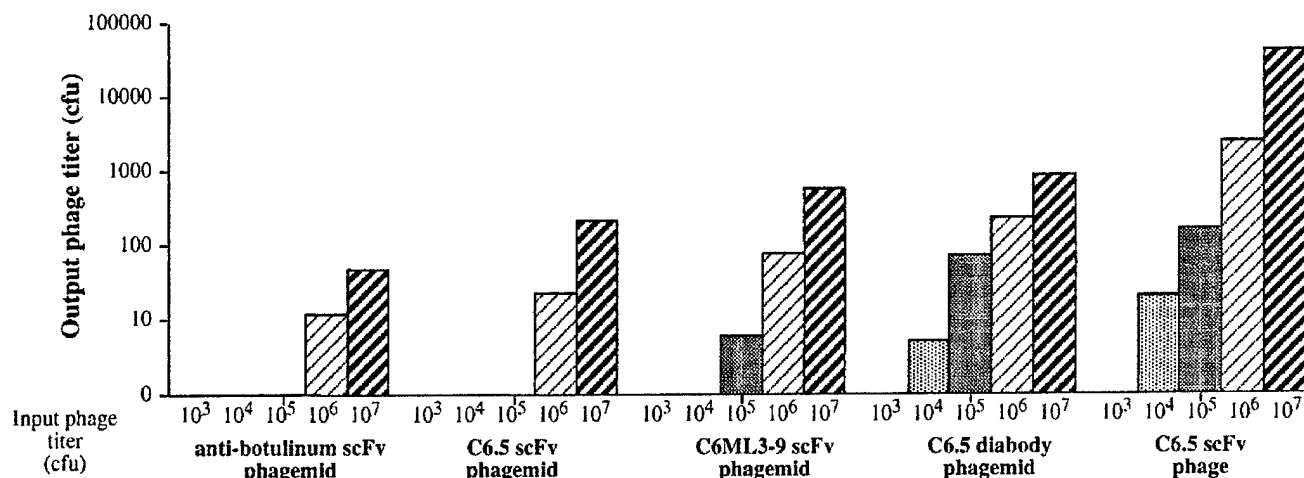


FIG. 6. Effect of phage concentration on the recovery of intracellular phage. Various concentrations of C6.5 scFv phagemid, C6ML3-9 scFv phagemid, C6.5 diabody phagemid or C6.5 scFv phage (input phage titer) were incubated with subconfluent SKBR3 cells grown in 6-well plates for 2 h at 37°C. Cells were treated as described in the legend to Fig. 4 including the trypsinization step and intracellular phage were titrated (output phage titer).

DISCUSSION

We demonstrate for the first time that phage displaying an anti-receptor antibody can be specifically endocytosed by receptor expressing cells and can be recovered from the cytosol in infectious form. The results demonstrate the feasibility of directly selecting internalizing antibodies from large non-immune phage libraries and identify the factors that will lead to successful selections. Phage displaying anti-ErbB2 antibody fragments are specifically endocytosed by ErbB2 expressing SKBR3 cells, can be visualized within the cytosol and can be recovered in an infectious form from within the cell. When monovalent scFv antibody fragments were displayed monovalently in a phagemid system, recovery of internalized phage was only 3.5- to 18-fold above background. Display of bivalent diabody or multivalent display of scFv in a phage vector increased recovery of internalized phage to 30- to 146-fold above background. This result is consistent with our studies of native monomeric C6.5 scFv and dimeric C6.5 diabody as well as studies of other monoclonal anti-ErbB2 antibodies where dimeric IgG but not monomeric Fab dimerize and activate the receptor and undergo endocytosis (7, 8). In fact it is likely that endocytosis of C6.5 and C6ML3-9 scFv phagemids reflect the small percentage of phage displaying two or more scFv (21). The importance of valency in mediating either high avidity binding or receptor crosslinking and subsequent endocytosis is confirmed by the only other report demonstrating specific phage endocytosis. Phage displaying approximately 300 copies of a high affinity Arg-Gly-Asp integrin binding peptide on pVIII were efficiently endocytosed by mammalian cells (11). Recovery of phage after endocytosis also increases the

specificity of cell selections compared to recovery of phage from the cell surface. Thus enrichment ratios for specific vs nonspecific surface binding range from 2- to 20-fold. These values are comparable to the approximately 10-fold enrichment reported by others for a single round of cell surface selection (25, 26). In contrast our enrichment ratios for specific vs non-specific endocytosis range from 3.5- to 146-fold.

Based on these results, selection of internalizing antibodies from phage antibody libraries would be most successful with either homodimeric diabodies in a phagemid vector or multivalent scFv using a phage vector. While no such libraries have been published, there are no technical barriers preventing their construction. Multivalent libraries would present the antibody fragment in the form most likely to crosslink receptor and undergo endocytosis. Antibodies from such libraries would need to be bivalent to mediate endocytosis. Alternatively, monomeric receptor ligands can activate receptors and undergo endocytosis, either by causing a conformational change in the receptor favoring the dimeric form or by simultaneously binding two receptors. Monomeric scFv that bound receptor in a similar manner could also be endocytosed. Thus selection of libraries of monovalent scFv in a phagemid vector could result in the selection of ligand mimetics that activate receptors and are endocytosed as monomers. Such scFv could be especially useful for the construction of fusion molecules for the delivery of drugs, toxins or DNA into the cytoplasm. Since antibodies which mediate receptor internalization can cause receptor downregulation and growth inhibition (8, 27-29), selection for endocytosable antibodies may also identify antibodies which directly inhibit or modulate cell growth.

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Internalizing antibodies and targeted cancer therapy: direct selection from phage display libraries

Ulrik B. Nielsen and James D. Marks

Antibody internalization is required for the success of many targeted therapeutics, such as immunotoxins, immunoliposomes, antibody–drug conjugates and for the targeted delivery of genes or viral DNA into cells. Recently, it has become possible to directly select antibody fragments from phage display libraries for internalization into mammalian cells. Here we review the therapeutic applications of internalized antibodies and describe how phage display enables the isolation of internalizing antibodies to novel or known targets.

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▼ Although antibodies show tremendous promise for the treatment of human malignancies, initial attempts to develop anti-tumor antibodies were generally unsuccessful. These failures were largely caused by the limitations of murine hybridoma technology including, for example, the immunogenicity of murine antibodies in humans. More recently, both improved understanding of tumor biology and advances in antibody engineering have made it possible to identify better tumor targets for antibody-based therapies and to generate less immunogenic humanized and human antibodies.

Studies of the molecular basis of tumorigenesis have identified cell surface receptors that are either: (1) tumor or lineage specific, such as CD20 (Ref. 1) and mutant forms of the epidermal growth factor receptor² (EGF receptor; see Glossary in Box 1), or (2) overexpressed in tumors, such as ErbB2 (Ref. 3). These cell surface receptors serve as ideal antibody targets.

Technologies for making antibodies

Advances in molecular cloning and antibody engineering have made it possible to convert rodent

monoclonal antibodies into chimeric antibodies (where the constant regions are human) or humanized antibodies (where the majority of the variable region sequence is also human). Such antibodies, especially those that are humanized, are significantly less immunogenic than rodent antibodies and can be consecutively administered without an increase in clearance or loss of efficacy.

Human antibodies

Several technologies have recently been developed to produce antibodies of entirely human origin. Transgenic mice harboring a portion of the human variable region (V) gene locus have enabled human antibodies to be produced using standard hybridoma technology⁴. Although this approach generates antibodies of entirely human sequence, it has similar limitations to traditional hybridoma technology. It relies on the availability of an immunogen and a natural immune response, and may yield only a limited number of antibodies, often directed to a few immunodominant

Box 1. Glossary

EGF Epidermal growth factor

Ig Immunoglobulin

V-gene Immunoglobulin variable region gene

scFv Single chain Fv antibody fragment

PEG Polyethylene glycol

IL Immunoliposomes

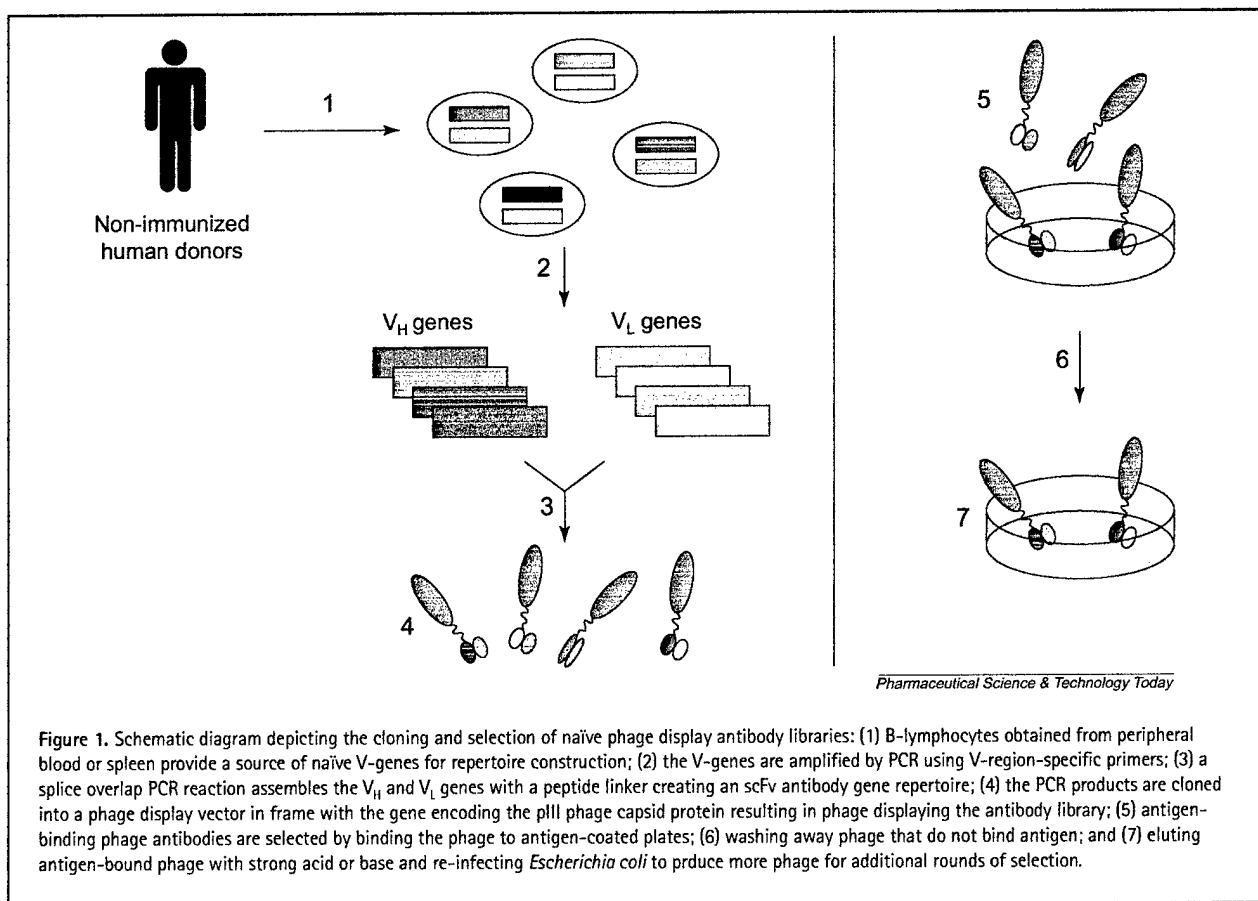
PE *Pseudomonas* exotoxin

RAIT Radioimmunotherapy

NCAM Neural cell adhesion molecule

PSMA Prostate-specific membrane antigen

CEA Carcinoembryonic antigen



epitopes. Phage display is another promising technology, which has produced antibody fragments that bind a wide variety of antigens, including several hitherto refractory immunogens⁵⁻⁷. Combinatorial antibody libraries are typically cloned from naïve repertoires of immunoglobulin (Ig) V-genes, such as IgM genes, from non-immunized donors and are displayed on phage (Fig. 1).

Gene fragments encoding the Ig heavy and light chain variable regions (V_H and V_L) are amplified from B-lymphocytes using PCR and are assembled as single-chain Fv antibody fragments (scFv). The assembled genes are inserted into a phage display vector in frame with the gene encoding the phage coat protein pIII. Following its introduction into *Escherichia coli*, the random combinatorial library of antibody fragments is displayed on phage. Antigen-specific antibodies can be selected from antibody libraries displayed on phage after one week and the antibody fragments typically express at high levels in *E. coli*⁸.

Another advantage of phage display is that the antibody genes are directly available for the subsequent genetic engineering of the antibody fragment, that is, to make fusion molecules⁹ or to improve antibody affinity¹⁰. The genetic engineering of antibody fragments has also enabled an extensive study of the physical properties of antibodies affecting the targeting

of human malignancies. Several parameters such as affinity¹¹, valence^{12,13}, charge¹⁴ and size¹⁵ have previously been shown to influence tumor targeting *in vivo*.

Antibody strategies for cancer therapy

As a result of the advances in antibody engineering and tumor biology, the first two antibodies approved for therapy of human cancers entered clinical practice: (1) Rituxan for non-Hodgkins lymphoma¹⁶ and (2) Herceptin for breast cancer¹⁷. These two antibodies were developed on the basis of their ability to bind cell surface receptors overexpressed on the target tumor (CD20 in the case of non-Hodgkins lymphoma and ErbB2 in breast cancer). Rituxan and Herceptin exert their therapeutic effects directly, either by inducing apoptosis (Rituxan^{18,19}) or by causing growth inhibition (Herceptin²⁰). Only a fraction of the antibodies generated against a known surface receptor, such as ErbB2, share this direct tumor inhibitory ability²¹. If the antibodies do not directly inhibit tumor growth, other strategies using the antibody to deliver a toxic payload must be used.

Many of these strategies rely on the ability of the antibody to bind to the surface receptor in a manner that induces

receptor-mediated endocytosis, resulting in the delivery of the cytotoxic agent into the cytosol. For example, anti-ErbB2 antibodies have been used to target doxorubicin-containing liposomes²² or *Pseudomonas* exotoxin (immunotoxin) in the interior of tumor cells^{9,23}. The use of antibodies to target non-viral gene therapy vectors also requires the antibody to induce receptor-mediated endocytosis in order to deliver the gene into the cell. Similar to growth inhibition, the majority of antibodies generated by immunization do not bind to receptors in a manner that triggers endocytosis^{21,24}, and it is therefore essential to select for antibodies that can elicit the desired response.

Exploiting receptor-mediated endocytosis for drug delivery

The endocytic pathway can be used by antibodies to deliver drugs into the cytosol. Typically, endocytosis plays a role in numerous cellular functions including antigen presentation, nutrient acquisition, receptor regulation and synaptic transmission. Endocytic pathways are also used by viruses, toxins and symbiotic microorganisms to gain entry into cells.

Internalization via clathrin-coated pits

One of the most well-characterized endocytic mechanisms is receptor-mediated endocytosis via clathrin-coated pits. The binding of ligands to receptors often leads to receptor aggregation, either by inducing a conformational change or by cross-linking receptors^{25,26}. In the case of the EGF receptor²⁷ and the Fc receptor type II (Ref. 28), the receptors subsequently concentrate in clathrin-coated pits resulting in endocytosis and clearance from the cell surface. Membrane proteins that are internalized in clathrin-coated pits contain targeting sequences in their cytoplasmic domains that interact with a variety of adaptor proteins, and clathrin, which directs the protein into these pits²⁹. The fate of the receptor–ligand complex after it is in the endocytic vesicle is dependent on the receptor. For example, the transferrin receptor enters the early endosomes from which it is rapidly recycled along with transferrin to the cell surface³⁰. By contrast, the EGF receptor is either recycled following dissociation of EGF or it accumulates in the late endosomes wherein it is degraded.

Drug delivery via internalizing antibodies

Antibodies and antibody fragments can deliver a variety of agents, such as drugs, genes, toxins and radionuclides, to target cells that express the antigen. The endocytosis of the antibody fragment to the interior of the cell can often increase the effect of the therapeutic agent. A major advantage of receptor-mediated endocytosis as a drug delivery route is that therapeutic agents can be delivered specifically into target cells that overexpress the receptor and thereby increase efficacy while reducing systemic

toxicity. The main disadvantage is that the therapeutic agent localizes to the endosomes, but it needs to escape from here into the cytoplasm in order to exert its pharmacological effects.

Immunoconjugates

Monoclonal antibodies directed to tumor-associated antigens have been chemically conjugated to a variety of drugs such as doxorubicin³¹ and more-toxic molecules such as enediynes³². Most immunoconjugates rely on the release of the drug from the antibody after it is in the endosome in order for it to exert its pharmacological activity in the cytosol or nucleus. Immunoconjugates that are internalized into cells by receptor-mediated endocytosis enter endosomes and lysosomes that contain a mildly acidic (pH 4–5) environment. This pathway offers a selective mechanism of drug release if drug carrier linkers have adequate differences in their rates of hydrolysis at lysosomal and systemic pH. Alternatively, the release of the drug from the antibody following internalization can take advantage of the metabolic potential of the endosomes and lysosomes³³.

Targeted gene delivery

To accomplish antibody-mediated gene delivery, the antibody must contain a domain that will complex or encapsulate the DNA vector. This can be a non-specific carrier domain, such as protamine³⁴, or natural protein domains that bind specific DNA sequences³⁵. Whatever the carrier, after targeting to the cell surface the DNA must enter the cell nucleus for gene expression. Receptor-mediated endocytosis has been investigated as a pathway for non-viral gene delivery into cancer cells; however, after it has entered the endosome, the gene must be released from the carrier and must enter the cytosol.

Research into how viruses escape from endosomes has resulted in the enhancement of gene expression using membrane-active peptides derived from viral domains³⁶, and translocation to the nucleus has been improved using nuclear localization signals³⁷. Also, cationic lipid–DNA complexes that efficiently escape the endosomes have been targeted to tumors. Such agents, however, are rapidly cleared from the circulation. Thus, the highest levels of activity are observed in ‘first pass’ organs, such as the lungs, spleen and liver. Viruses, which inherently escape from the endosome, have also been targeted with antibodies. Engineered viruses, however, can generate an immune response that can compromise transfection efficiency on subsequent injections. In addition, the natural wild-type tropism must be attenuated to obtain tumor target specificity. Eventually, a better understanding of endosomal escape will lead to targeted gene delivery constructs that achieve high gene expression without the potentially harmful toxicity associated with viral gene delivery.

Immunoliposomes

Several liposomal drugs, such as the liposome-encapsulated doxorubicin, have proven to be effective against cancer in clinical trials³⁸. The steric stabilization of liposomes with polymers such as polyethylene glycol (PEG) have increased the circulation time by reducing the rate of reticuloendothelial clearance and increasing the uptake by tumors³⁹. The coupling of antibodies to liposomes to form immunoliposomes (ILs) shows promise for increasing the efficacy of liposomal drugs against solid tumors and leukemia by specific interaction with the tumor cells. In early studies, a strong association between enhanced growth inhibition and liposome internalization was observed *in vitro*⁴⁰; however, the binding of ILs displaying antibodies is not always followed by internalization⁴¹. Since these studies, numerous investigations have demonstrated that the cytotoxicity of the liposome-encapsulated drug increases when the liposome carrier is internalized into the target cell^{40,42-45}. Enhanced efficacy *in vivo* also appears to depend on internalization. When the monoclonal anti-ErbB2 antibody N-12A5 was coupled to sterically stabilized liposomes, no increased efficacy over untargeted liposomes was observed⁴¹. By contrast, when an internalizing anti-ErbB2 Fab was used for IL construction, greatly enhanced efficacy in a mouse xenograft model⁴⁶ was observed owing to enhanced IL uptake into tumor cells (D. Kirpotin *et al.*, unpublished).

Immunotoxins

Immunotoxins are attractive candidates for cancer therapy because they combine the specificity of tumor-cell-reactive antibodies with the high cytotoxic potency of naturally occurring toxins^{9,23}. *Pseudomonas* exotoxin (PE) is frequently used for immunotoxin construction. PE and related toxins consist of three regions involved in binding, translocation and activity. The translocation domain is believed to actively transport the active domain from the endosome into the cytosol. This makes toxins such as PE well suited for targeting by receptor-mediated endocytosis because this is the pathway that the toxin naturally transits before entering the cytosol, where it efficiently inhibits protein synthesis. Indeed, immunotoxins have shown efficacy in several clinical trials, particularly for the treatment of hematological tumors⁴⁷. Endocytosis of the antigen-immunotoxin complex appears to be the most important determinant of *in vitro* cytotoxicity. Other factors, such as the extent of cell binding and the number of cell surface antigens, appear to affect cytotoxicity only to the degree that they influence endocytosis⁴⁸.

Radionuclide antibody conjugates

Radioimmunotherapy (RAIT) or immunoscintigraphy using systemically administered antibodies linked to radionuclides is

a promising approach to the treatment and diagnosis of cancer. It is not immediately obvious that antibody internalization is advantageous for RAIT and immunoscintigraphy. Following internalization, radioiodinated antibodies are usually degraded and dehalogenated intracellularly⁴⁹, leading to the conclusion that non-internalized antibodies would be superior. However, the intracellular degradation of radiolabelled antibodies and the subsequent secretion of radioactive iodine does not seem to prevent the accumulation of intracellular radioactivity. Indeed, the accumulation and retention of radioactivity in the tumor tissue, owing to the internalization of radiolabelled antibody, improved the immunoscintigraphy of xenografts in nude mice⁵⁰. Furthermore, dehalogenation in the cell only takes place when iodine nuclides are attached to tyrosine residues using Trouts reagent⁵¹ (other radionuclides such as ¹¹¹In or chelated nuclides can also be used). In RAIT, internalization of the antibodies used for targeting are also advantageous. The emission characteristics of the radioisotope are critical in determining the appropriate radiation dose to the tumor compared with normal organs. If antibodies internalize and transport low-energy electron-emitting isotopes close to the tumor cell nucleus, an improved therapeutic advantage can be achieved. In the case of Auger emitters such as ¹²⁵I, lower toxicity is observed. This is probably caused by the short path length of their low-energy electrons, which can reach the nuclear DNA only if the antibody is internalized⁵².

For several other antibody-based strategies, internalization can be prohibitive. For instance, bi-specific antibodies and immune-stimulatory fusion proteins require interaction on the cell surface with cells of the immune system, thus making internalization undesirable. Likewise, internalization is not desired for antibody-directed enzyme prodrug therapy in which an antibody-bound enzyme is localized to the cell surface where it enzymatically converts a prodrug.

Factors that influence antibody internalization

Several approaches have been used to develop antibody-based delivery systems that use endocytosis as a point of entry into cells. The limitations of endocytosis as an entry point for drugs into cells depends on the: (1) type of receptor, (2) antigen density, (3) epitope, (4) rate of internalization, (5) release of the therapeutic molecule from the endosome, and (6) re-expression of the antigen on the cell surface (Fig. 2).

Several antibodies to cell surface receptors, such as the EGF receptor^{53,54}, ErbB2 (Refs 55,56) and transferrin receptor⁵⁷, induce internalization. Other cell surface molecules have also been shown to mediate antibody internalization (although often at a slower rate). These include the neural cell adhesion molecule⁵⁰ (NCAM), prostate-specific membrane antigen⁵⁸ (PSMA), carcinoembryonic antigen⁵⁹ (CEA) and mucins⁶⁰. The

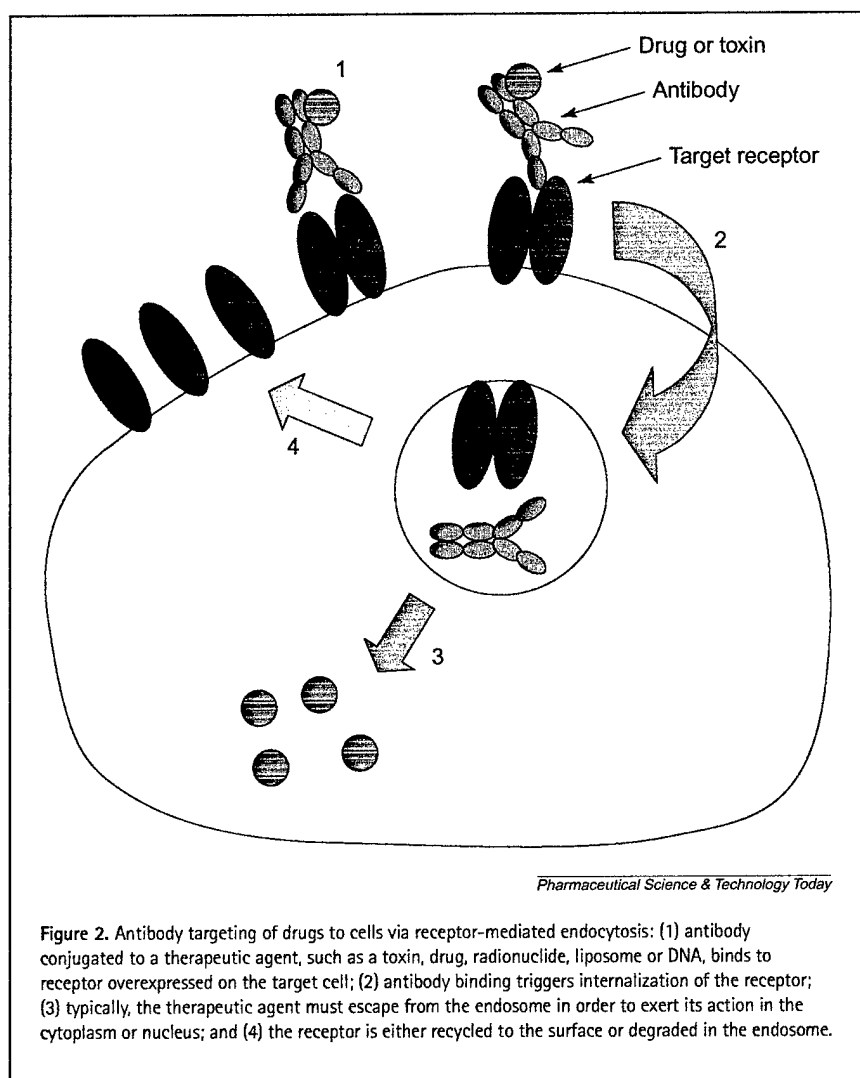


Figure 2. Antibody targeting of drugs to cells via receptor-mediated endocytosis: (1) antibody conjugated to a therapeutic agent, such as a toxin, drug, radionuclide, liposome or DNA, binds to receptor overexpressed on the target cell; (2) antibody binding triggers internalization of the receptor; (3) typically, the therapeutic agent must escape from the endosome in order to exert its action in the cytoplasm or nucleus; and (4) the receptor is either recycled to the surface or degraded in the endosome.

therapeutic potential of antibodies and antibody-targeted drugs has been correlated with antigen density on the target cell surface^{61,62}. A high density of cell surface receptors permits more antibodies to be concentrated on target cells, which consequently results in greater pharmacological effectiveness⁶². The choice of antigen is a key factor for targeting malignancies because, to a large extent, it determines the rate of internalization and intracellular routing^{43,63}. Indeed, the rate of internalization plays a key role in predicting the cytotoxicity of drug or toxin conjugates^{64,65}.

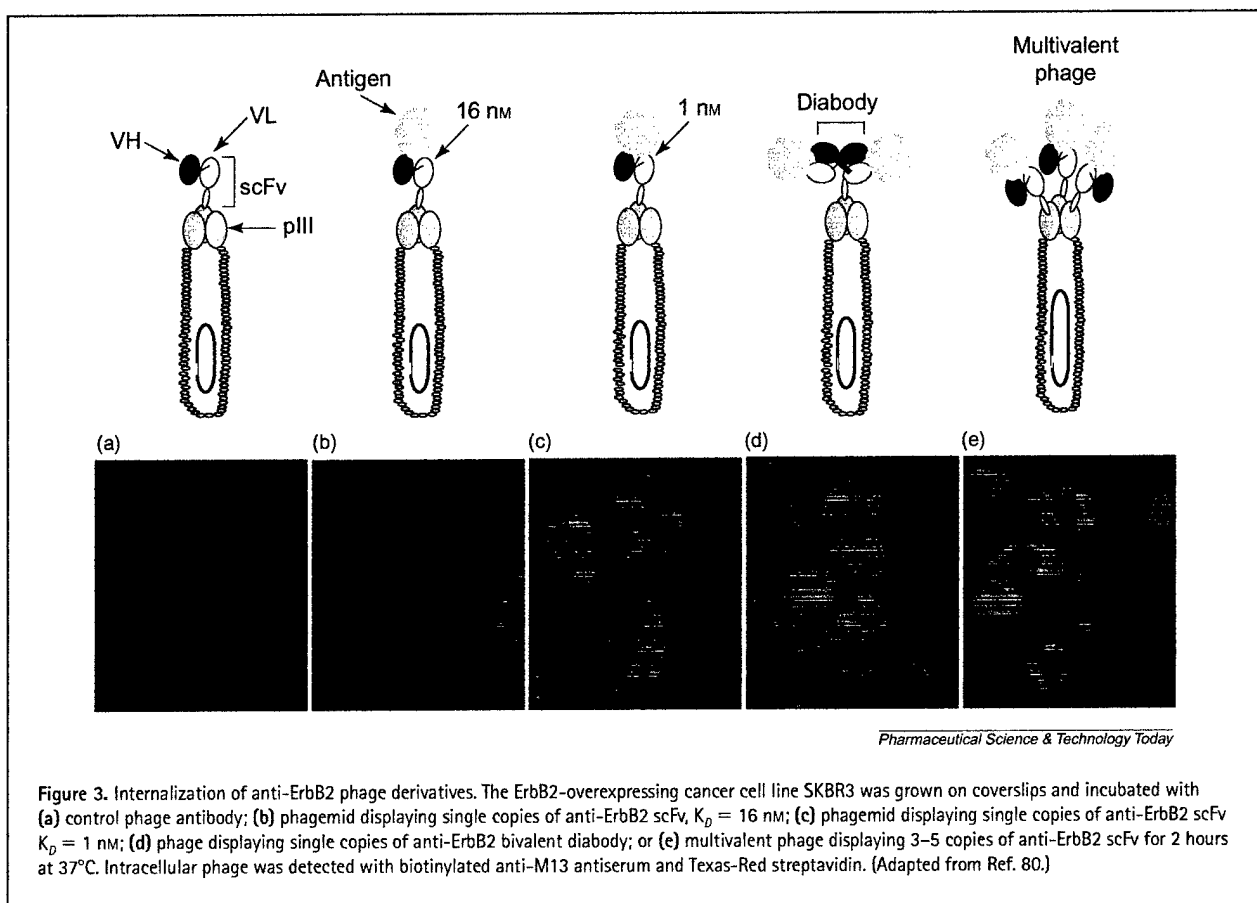
However, not all antibodies binding to internalizing receptors are rapidly internalized^{21,56}. It appears that the epitope recognized by the targeting antibody influences the rate of internalization^{65,66}. Frequently, the antibody mimics the natural ligand. For instance, some internalizing antibodies against the EGF receptor cause tyrosine phosphorylation and, in some

cases, also mimic the mitogenic effects of EGF^{54,67}. Tyrosine phosphorylation of the EGF receptor, however, is not a requirement for antibody internalization⁶⁸. Similar discrepancies have been reported for the activities of internalizing anti-ErbB2 monoclonal antibodies^{56,69}.

Most of the investigations carried out on the internalization of antibodies did not address the role of multi-valency in antibody internalization. For many antibodies, however, bivalency may be mandatory for internalization. When monovalent Fab fragments of several anti-ErbB2 antibodies were tested for internalization, the fragments were not internalized⁷⁰. Similar observations were reported for the Fab portion of an anti-EGF receptor antibody⁵³. Further, increasing the valency of antibodies can also increase their internalization. For instance, internalization of IgG aggregates by polymorphonuclear neutrophils varies with the size of the aggregates⁷¹. Thus, caution must be used when designing recombinant antibody targeted drugs with a monovalent binding site, such as scFv or Fab, to ensure efficient internalization.

Screening antibodies for internalization

The most common method for monitoring the internalization of ligands and antibodies into cells uses a low pH buffer (typically glycine-HCl, pH 2.8) to dissociate the surface-bound antibody. However, reports from several groups indicate that this buffer, in some cases, only partially dissociates antigen-antibody complexes and therefore can introduce major inaccuracies in internalization experiments^{72,73}. Alternatively, antibodies can be biotinylated with NHS-SS-biotin and incubated with live cells. Following the specific reduction of biotin groups on cell-surface-bound antibodies with reducing agents, internalization can be quantitated by immunoblotting⁵⁸. However, the accuracy of this method also relies on the complete removal of biotin from the cell-surface-bound antibody. All of the above-mentioned screening methods are laborious, allowing only a limited number of different antibodies to be screened for internalization.



Direct selection of internalizing antibodies from phage display libraries

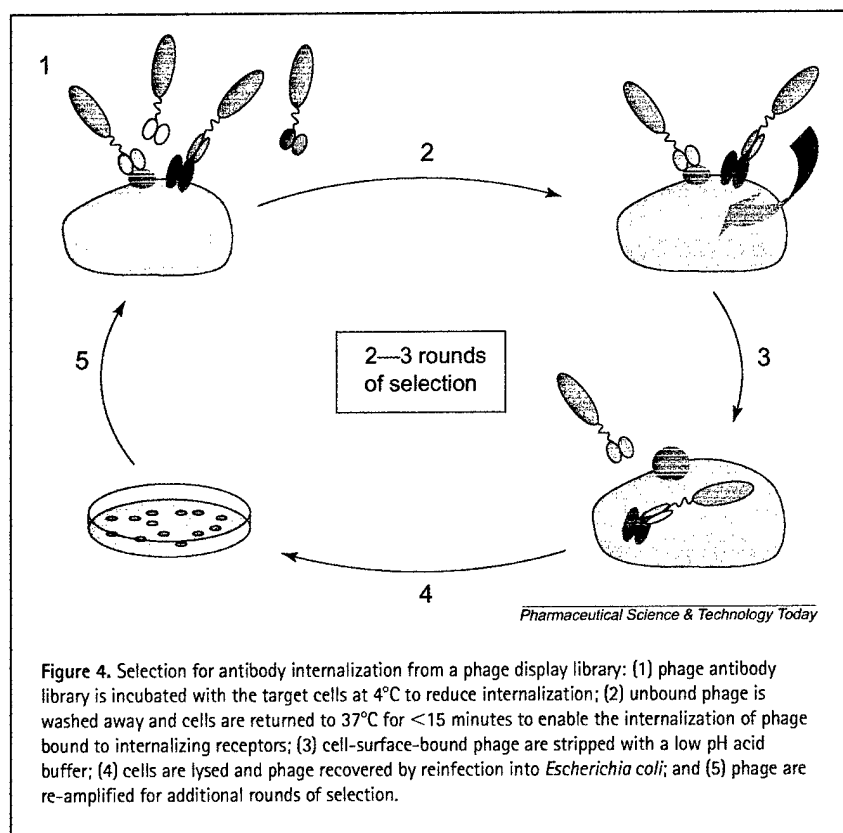
Because phage antibody isolation takes place *in vitro*, selection procedures can be manipulated to select for antibodies with desired physical or biological activities. Recently, the direct selection of peptides and antibody fragments binding cell surface receptors from filamentous phage libraries by the incubation of phage libraries with a target cell line has been demonstrated^{6,74–77}. This has led to an increase in the number of potential targeting molecules. However, the isolation of cell-type-specific antibodies from naïve libraries has been difficult because selections often result in the isolation of antibodies to common cell-surface antigens⁷⁸.

Phage internalization into mammalian cells

The ability of bacteriophage displaying peptides to undergo receptor-mediated endocytosis^{75,79} indicates that phage antibody libraries might be selected not only for their cell binding but also for their internalization into mammalian cells. Unlike peptide phage libraries, however, phage antibody libraries typically display monomeric scFv or Fab antibodies fused to pIII as

single copies on the phage surface (phagemid libraries). It has been hypothesized that such monovalent display is unlikely to lead to efficient receptor cross-linking and phage internalization. To determine the feasibility of selecting internalizing antibodies and to identify the factors responsible for phage internalization, the C6.5 phage antibody has been investigated. C6.5 scFv binds the growth factor receptor ErbB2, which is overexpressed in many solid tumors. Similar to the majority of antibodies, monovalent C6.5 scFv is only minimally internalized, although the bivalent diabody is efficiently endocytosed⁸⁰. Investigations have also been carried out on the phagemid displaying single copies of C6.5 scFv⁸¹, phagemid displaying a 16-fold higher affinity mutant of C6.5 scFv¹⁰, phagemid displaying single copies of the bivalent C6.5 diabody¹² and phage displaying multiple copies of C6.5 (Fig. 3). For these studies, the phage were incubated with live cells at 37°C, the surface-bound phage was removed by acid washing and the endocytosed phage recovered by cell lysis.

The internalization of monovalent C6.5 scFv was only four-fold greater than the internalization of a non-specific anti-botulinum phage antibody (background internalization).



Display of the 16-fold higher affinity C6.5 mutant increased internalization to 16-fold above background. Endocytosis was greatest when the phage antibody was multivalent, prepared either by using the bivalent diabody or by display on a phage vector. The uptake of multivalent phage increased to 30-fold (diabody) and 146-fold (phage vector) above background. For any display format, the enrichment ratio above background was much greater for internalized phage compared with phage recovered from the cell surface (only two-fold to 20-fold above background). The result of the uptake of the different phage antibodies as determined by fluorescence microscopy agreed with uptake determined by phage titering (Fig. 3). These experiments demonstrated the feasibility of directly selecting internalizing antibodies from phage libraries and indicated that selection is most efficient for bivalent diabodies or scFv displayed multivalently on phage.

Selection of internalizing antibodies from phage display libraries

The strategy described above was used to directly select from a large naïve phage antibody library⁷ a scFv capable of triggering endocytosis into breast tumor cells upon receptor binding⁸² (Fig. 4). This library displays single copies of monovalent scFv

using a phagemid vector. Although our results described above indicate that this is not the optimal display format for internalization selections, no diabody or phage based naïve libraries currently exist.

After three rounds of selection, greater than 50% of the scFv analysed bound to the selecting cell line. The further characterization of several of these antibodies identified two that bound ErbB-2 (F5 and C1) and three that bound the transferrin receptor. Interestingly, both ErbB-2 and the transferrin receptor are rapidly internalized and are specific markers for several cancers⁸³. Both the phage antibodies and the native purified scFv were rapidly endocytosed into cells expressing the appropriate receptor. The scFv that bind ErbB2 and the transferrin receptor did not spontaneously dimerize and do not require dimerization in order to undergo internalization⁸². The internalization of the anti-ErbB2 scFv F5 was compared with the C6.5 scFv, which was selected on recombinant ErbB2 protein using tradi-

tional panning strategies⁸¹. The antibodies recognize different epitopes on ErbB2 with comparable affinities; however, C6.5 scFv does not significantly internalize into ErbB2 as monomeric scFv, whereas F5 scFv does.

Using this strategy of selection for internalization into tumor cell lines, internalizing antibodies to ErbB2, transferrin receptor and EGF receptor have now been isolated. In addition, hundreds of antibodies to unidentified targets have been isolated, several of which appear to be overexpressed on breast cancer cells. Given the nature of the antigens identified to date, the identification of novel antigens from these selections using expression cloning and protein microsequencing are currently being pursued. It is envisaged that selection on other cell types will identify other cell-specific markers because endocytosed receptors are likely to be associated with specific cell functions, such as growth factor and nutrient transport receptors on cancer cells or Fc receptors on cells of the immune system. This approach can also be used to generate internalizing antibodies to known receptors by transfecting the receptor into an appropriate cell line and performing selections as described above. It might also be possible to use internalization as a surrogate marker to identify desirable biological effects of the antibody, for example, apoptosis or growth inhibition. Indeed,

a significant growth inhibitory effect of the anti-transferrin scFv identified in the selection on cancer cells was observed⁸². Thus, antibodies selected using this approach might have a direct therapeutic effect in addition to the ability to deliver drugs into the cytosol.

Human scFv antibodies selected from a phage display library for internalization into tumor cells can readily be used as targeting molecules for drug delivery. For example, one of the scFv against ErbB2 was conjugated to the surface of commercial liposomal doxorubicin converting it into fully functional doxorubicin-loaded anti-ErbB2 immunoliposomes (U.B. Nielsen et al., unpublished). The resulting ILs have superior efficacy in an ErbB2 overexpressing mouse xenograft model compared with untargeted liposomal doxorubicin. Because of the entirely human origin of the scFv, it is likely that the resulting ILs will be non-immunogenic in humans.

Conclusion

It is envisaged that the selection for internalization methodology will be generally applicable to generate scFv capable of delivering liposomes or other agents into a wide variety of tumor cells, such as prostate and ovarian cancers. In addition, the generation of multivalently displayed antibody fragment libraries (either as diabodies or on phage vectors) should greatly increase the number of internalizing antibodies generated using this approach. Compared with scFv isolated from monovalently displayed libraries, these antibody fragments might need to be used multivalently (as on liposomes) in order to be internalized. Alternatively, they might be useful as monovalent scFv to target the cell surface for bispecific therapeutic approaches because they are not likely to be endocytosed in that format.

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Approval...

Schering-Plough (Madison, NJ, USA) have announced the recommendation of approval by the EU's Committee for Proprietary Medicinal Products (CPMP) of the European Agency for the Evaluation of Medicinal Products (EMEA) for the use of CAELYX (pegylated liposomal doxorubicin hydrochloride) in the treatment of advanced ovarian cancer. It is proposed that CAELYX will be administered intravenously once every four weeks for as long as the disease does not progress and the treatment is tolerated by the patient.

Selection of Tumor-Specific Internalizing Human Antibodies from Phage Libraries

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Antibody internalization into the cell is required for many targeted therapeutics, such as immunotoxins, immunoliposomes, antibody-drug conjugates and for targeted delivery of genes or viral DNA into cells. To generate directly tumor-specific internalizing antibodies, a non-immune single chain Fv (scFv) phage antibody library was selected on the breast tumor cell line SKBR3. Internalized phage were recovered from within the cell and used for the next round of selection. After three rounds of selection, 40% of clones analyzed bound SKBR3 and other tumor cells but did not bind normal human cells. Of the internalizing scFv identified, two (F5 and C1) were identified as binding to ErbB2, and one (H7) to the transferrin receptor. Both F5 and H7 scFv were efficiently endocytosed into SKBR3 cells, both as phage antibodies and as native monomeric scFv. Both antibodies were able to induce additional functional effects besides triggering endocytosis: F5 scFv induces downstream signaling through the ErbB2 receptor and H7 prevents transferrin binding to the transferrin receptor and inhibits cell growth. The results demonstrate the feasibility of selecting internalizing receptor-specific antibodies directly from phage libraries by panning on cells. Such antibodies can be used to target a variety of molecules into the cell to achieve a therapeutic effect. Furthermore, in some instances endocytosis serves as a surrogate marker for other therapeutic biologic effects, such as growth inhibition. Thus, a subset of selected antibodies will have a direct therapeutic effect.

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Keywords: receptor mediated endocytosis; ErbB2; phage antibody library; single chain Fv; tumor targeting

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Abbreviations used: scFv, single chain Fv; cfu, colony forming units; EGFR, epidermal growth factor receptor; TEA, triethylamine; ELISA, enzyme linked immunosorbent assay; ECD, extracellular domain; CHO, Chinese hamster ovary cells; IMAC, immobilized metal affinity chromatography; HRP, horseradish peroxidase; TfR, transferrin receptor; V_H, immunoglobulin heavy chain variable domain; V_L, immunoglobulin light chain variable domain; FBS, fetal bovine serum; FCS, fetal calf serum; EGF, epidermal growth factor; RT, room temperature; BSA, bovine serum albumin; FACS, fluorescent activated cell scanning; HBS, Hepes-buffered saline.

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Introduction

Antibody internalization into the cell is required for many targeted therapeutics, such as immunotoxins (Altenschmidt *et al.*, 1997), immunoliposomes (Kirpotin *et al.*, 1997), antibody-drug conjugates and for targeted delivery of genes or viral DNA into cells (Fominaya & Wels, 1996). This can be accomplished by taking advantage of normal receptor biology: ligand binding causes receptor activation *via* homo- or heterodimerization, either directly for a bivalent ligand or by causing a conformational change in the receptor for monovalent ligand, and receptor-mediated endocytosis (Ullrich & Schlessinger, 1990). Antibodies can mimic this process, stimulate endocytosis, become internalized and deliver their payload into the cell. In general, this requires a bivalent antibody capable of mediating receptor dimerization (Heldin, 1995; Yarden, 1990). In addition, the effi-

ciency with which antibodies mediate internalization differs significantly depending on the epitope recognized (Hurwitz *et al.*, 1995; Yarden, 1990).

Currently, antibodies which trigger internalization are identified by screening antibodies derived by either hybridoma or phage antibody technology. However, this usually involves examining antibodies recognizing specific targets, and while it may take the biology of the target into account, it takes no account of the biology of the antibody that is triggering receptor endocytosis. Antibodies which trigger receptor endocytosis could be directly selected from large non-immune phage libraries (Marks *et al.*, 1991; Sheets *et al.*, 1998) by recovering infectious phage particles from within cells after receptor-mediated endocytosis, as reported for peptide phage libraries (Hart *et al.*, 1994; Barry *et al.*, 1996). Unlike the multivalently displayed peptide phage libraries, however, phage antibody libraries typically display monomeric single chain Fv (scFv) or Fab antibody fragments fused to pIII as single copies on the phage surface using a phagemid system (de Haard *et al.*, 1999; Knappik *et al.*, 2000; Marks *et al.*, 1991; Nissim *et al.*, 1994; Sblatero & Bradbury, 2000; Sheets *et al.*, 1998; Vaughan *et al.*, 1996). To determine the feasibility of selecting internalizing antibodies, we previously studied the human scFv C6.5 which binds the growth factor receptor ErbB2 (Schier *et al.*, 1995). Using wild-type C6.5 scFv displayed monovalently in a phagemid system, we demonstrated that anti-ErbB2 phage antibodies can undergo receptor-mediated endocytosis, albeit with very low efficiency and enrichment ratios (Becerril *et al.*, 1999). The low efficiency reflects the expectation that a monomeric binding unit is unable to mediate receptor cross-linking and phage internalization. Since C6.5 was selected for binding to native protein, we reasoned that if scFv were able to mediate internalization in the monomeric form, the most effective way to identify them would be by direct selection from a large non-immune phage library by recovery of infectious phage particles from within tumor cells. Here we report the successful selection and characterization of such tumor-specific internalizing antibodies.

Results

Selection of internalizing phage antibodies

For selections, phage were prepared from a 7.0×10^9 member human scFv phage antibody library (Sheets *et al.*, 1998). To select for internalizing phage antibodies, 5×10^6 subconfluent adherent SKBR3 breast tumor cells were incubated with 1×10^{12} colony forming units (cfu) of phage in the presence of normal human fibroblasts in suspension for 1.5 hours. This step was performed at 4°C to allow phage binding without internalization. The fibroblasts were used to deplete the library of phage antibodies which bound to antigens common to SKBR3 cells and fibroblasts. Two different

sets of selections were performed in parallel. For one set of selections, fibroblast depletion was performed during each round of selection. For the second set of selections there was no fibroblast depletion in the first round to avoid the potential loss of rare phage antibodies which bound to antigens that were quantitatively but not qualitatively different between the selecting and depleting cell lines. After phage binding, the cells were washed extensively with phosphate-buffered saline (PBS) to remove non-specifically or weakly bound phage. Cells were then incubated at 37°C for 15 minutes to allow endocytosis of surface-bound phage. Fifteen minutes was chosen, since it is long enough to allow ligand-induced internalization of receptors like epidermal growth factor receptor (EGFR) and ErbB2 and short enough to avoid phage degradation within the cell, which would impair the recovery of infectious phage (Becerril *et al.*, 1999). To recover phage from within the cell, the cells were stripped three times with a low pH glycine buffer to remove phage bound to the cell surface, trypsinized and washed with PBS to remove phage bound in the extracellular matrix or to the culture plate, and finally lysed with high pH triethylamine (TEA). The cell lysate containing phage recovered from within the cell was used to infect *Escherichia coli* TG1 to prepare phage for the next round of selection. A total of three rounds of selection were performed. Selections were monitored by titering: (1) the number of phage bound to the cell surface in the first low pH glycine wash (wash 1); and (2) the number of endocytosed phage recovered from within the cell (cell lysate) (Table 1). For both selection strategies (\pm depletion on fibroblasts in the first round), the titer of phage bound to the cell surface increased only four- to tenfold after three rounds of selection while the titer of phage recovered in the cell lysate increased 100 to 200-fold (Table 1, and data not shown for selection without depletion in round 1). This resulted in more than a 100-fold increase in the number of endocytosed phage recovered per cell, from 0.01 phage/cell up to 3.75 phage/cell (Table 1). These data suggest that phage were selected on the basis of endocytosis into SKBR3 cells.

Initial characterization of phage antibodies

Antibodies binding SKBR3 cells were identified by cell ELISA using native soluble scFv expressed from randomly picked single colonies from the second and third rounds of selection. When depletion was included in each round of selection, 11/94 (11.7%) of the clones bound SKBR3 cells after two rounds of selection and 55/135 (40.7%) bound SKBR3 cells after three rounds of selection (Table 2). All positive clones gave no signal above background on fibroblasts in a cell ELISA assay, indicating that the library had been efficiently depleted of antibodies common to fibroblasts and SKBR3. The frequency of SKBR3 ELISA positive clones was similar when depletion was not

Table 1. Results of phage antibody library selection on SKBR3 cells

Round	Number of fibroblasts used for depletion	Phage output			Phage input/output ratios ($\times 10^{-6}$)		Phage output/cell		
		Phage input	Wash 1 (cell surface)	Cell lysate	Wash 1 (cell surface)	Cell lysate	Number of cells	Number of phage/cell (cell surface)	Number of phage/cell (intracellular)
1	0	3.0E + 12	3.6E + 06	9.7E + 04	120	3.2	ND	ND	ND
2	5.0E + 06	2.7E + 12	1.1E + 06	5.0E + 04	40	1.8	3.4E + 06	0.32	0.01
3	4.5E + 06	8.4E + 12	4.4E + 07	1.0E + 07	523	119	3.9E + 06	11.3	2.7
1	5.0E + 06	3.0E + 12	3.8E + 06	3.5E + 04	126	1.2	2.7E + 06	1.4	0.01
2	4.5E + 06	1.0E + 13	2.0E + 06	1.2E + 05	20	1.2	3.3E + 06	0.6	0.04
3	1.2E + 06	1.7E + 13	1.3E + 07	7.5E + 06	76	44	2.0E + 06	6.5	3.8

ND, not determined.

included in the first round; however, the majority of these antibodies (greater than 90%) also bound fibroblasts (data not shown). Thus, depletion during each round of selection was essential for the selection of cell-specific antibodies. Subsequent characterization focused only on the library which had been depleted during each round of selection.

Since SKBR3 cells are known to express high levels of the internalizing receptor ErbB2, bacterial supernatants containing soluble scFv were screened for binding to ErbB2 extracellular domain (ECD) by ELISA. After three rounds of selection, 29/135 clones (21%) bound ErbB2 ECD (Table 2). This represents approximately 50% of the antibodies which bound SKBR3 cells. The number of unique ErbB2 binders was determined by PCR fingerprinting of the 29 ELISA positive clones followed by DNA sequencing. Two unique ErbB2-binding human scFv (F5 and C1) were identified. Neither of these antibodies was similar in sequence to the 14 anti-ErbB2 scFv obtained selecting the same phage antibody library on purified recombinant ErbB2 ECD (Sheets *et al.*, 1998).

Further characterization of ErbB2 binding antibodies

F5 and C1 phage antibodies were analyzed for binding to a panel of cell lines whose ErbB2

Table 2. Frequency of binding clones after selection of a phage library on SKBR3 cells

Round of selection	ErbB2 positive phage (%) ^a	SKBR3 positive phage (%) ^b
1 (First round no depletion)	ND	ND
2	0.50	ND
3	1.10	ND
1 (First round depleted)	ND	ND
2	5.10	11.70
3	21.40	40.70

ND, not determined.

^a As determined by ELISA of 96 random clones on recombinant ErbB2 ECD.

^b As determined by ELISA of 96 random clones on SKBR3 cells.

expression had been described (Lewis *et al.*, 1993). They both stained SKBR3 and SKOV3 cells strongly, stained MCF7 cells weakly and did not stain fibroblasts and the normal breast cell line Hs578Bst (Table 3). The same profile was observed using phage displaying the C6.5 scFv that recognizes ErbB2 (Schier *et al.*, 1995) and the anti-ErbB2 monoclonal antibody 4D5 (Sarup *et al.*, 1991). F5 and C1 did not stain Chinese hamster ovary (CHO) cells or CHO cells transfected with the EGF receptor but did stain CHO cells transfected with ErbB2 (not shown). For subsequent studies, the F5 and C1 scFv genes were subcloned into a vector which fused a C-terminal hexahistidine sequence, expressed from *Escherichia coli* TG1 and purified by immobilized metal affinity chromatography (IMAC). Gel filtration analysis indicated that both F5 (Figure 1) and C1 existed exclusively as monomeric scFv with no apparent spontaneous dimerization or aggregation as seen with some scFv

Table 3. Characterization of phage antibody binding to a panel of cells by flow cytometry

Primary antibody	Cell type				
	SKBR3	MCF7	SKOV3	LNCaP	Fibroblasts
A. ErbB2 positive scFv-phage					
C6.5	526 ^a	16	670	nd	1
F5	4867	123	5839	nd	11
C1	858	0	566	nd	0
4D5 (Mab)	600	29	586	nd	2
B. ErbB2 negative scFv-phage					
3TE3	1056	1002	416	nd	130
H7	4003	1219	945	nd	93
3TB5	225	301	336	199	9
2TF5	1973	495	805	nd	0
3TH8	153	1	353	1	0
3TG5	469	80	714	82	3
3TF12	611	83	31	233	7
2TB4	138	3	1	nd	1
C2-1	181	8	81	nd	1
3GD8	103	6	1154	45	0

nd, not determined.

^a Results are expressed in mean fluorescent intensity (MFI) minus background fluorescence.

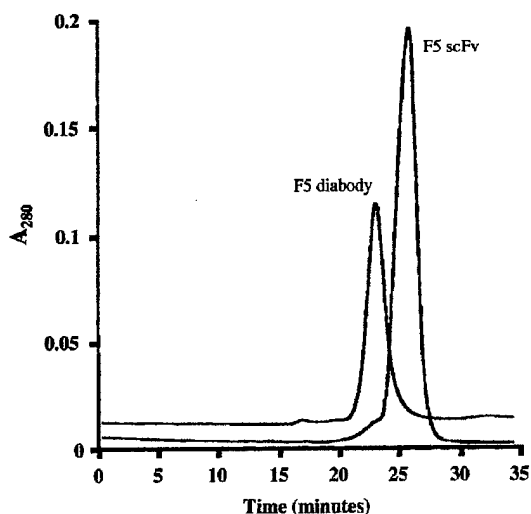


Figure 1. Gel filtration analysis of purified F5 scFv and diabody. F5 scFv and diabody were purified by IMAC and analyzed by gel filtration on a Sephadex 200 column. The mobility of the scFv was consistent with a molecular mass of 25 kDa, with no evidence of dimerization (the mobility of the diabody peak).

(Griffiths *et al.*, 1993). F5 bound recombinant ErbB2 with a $K_D = 1.6 \times 10^{-7}$ M as measured by surface plasmon resonance (SPR) in a BIAcore, and bound ErbB2 on SKBR3 cells with a $K_D = 1.36 \times 10^{-7}$ M. The K_D value of C1 was at least tenfold lower. Interestingly, these two scFv recognized the same epitope on ErbB2 as determined by competition ELISA (Figure 2(a)). This epitope was different than the epitope recognized by the human scFv phage antibody C6.5 and the murine monoclonal antibody 4D5 (Figure 2(a) and (b)). Since F5 and C1 recognized the same epitope, subsequent characterizations were performed using the higher affinity F5. F5 detected a band of the appropriate size for ErbB2 in a Western blot of the SKBR3 cell lysate (Figure 2(c)) and could immunoprecipitate ErbB2 from a SKBR3 cell lysate (Figure 2(d)).

Further characterization of non-ErbB2 binding antibodies

To characterize further the specificity of non-ErbB2 binding antibodies, phage were analyzed for binding to a panel of tumor and normal cell lines using flow cytometry. Phage were used for these studies rather than native soluble scFv because phage generate stronger signals due to signal amplification that results from the multiple copies of the major coat protein pVIII. To identify unique antibodies for flow cytometry studies, the scFv gene was PCR amplified from 18 SKBR3 positive and ErbB2 ECD negative clones and fingerprinted using the frequently cutting restriction enzyme *Bst*NI. Ten unique fingerprint patterns were identified

representing ten unique antibodies. Phage were prepared from each different pattern and used to stain a panel of human cell lines (normal human fibroblasts, the breast tumor cell lines SKBR3 and MCF7, the ovarian tumor cell line SKOV3 and the prostate tumor cell line LNCaP). All ten phage antibodies stained SKBR3 cells better than fibroblasts as measured by flow cytometry (Table 3). Some phage antibodies stain all tumor cell lines (clones 3TE3, H7, 3TB5 and 2TF5) with a high intensity while others stain only subsets of cells (SKBR3 and SKOV3 cells: clones 3TH8 and 3TG5, SKBR3 and LNCaP cells: clone 3TF12, SKBR3 cells: clones 2TB4 and C2-1, or SKOV3 cells: clone 3GD8). We selected one of these phage antibodies that bound all tumor cells analyzed (H7) for further characterization.

H7 phage antibody binds the transferrin receptor

The H7 scFv gene was subcloned, expressed and purified by IMAC as described above. Like F5 and C1 scFv, gel filtration analysis indicated that H7 existed exclusively as monomeric scFv with no apparent spontaneous dimerization or aggregation. To identify the receptor bound by H7 scFv, initially we attempted to detect an immunoreactive band by Western blotting. However, no immunoreactive bands were apparent in a blot of SKBR3 cell lysate (Figure 2(c)). We therefore used the H7 scFv to immunoprecipitate membrane biotinylated SKBR3 cell extracts. For this experiment, H7 scFv was bound to Ni-NTA agarose, and biotinylated cell extracts were incubated with the loaded agarose beads. Bound immunocomplexes were eluted using imidazole, the eluted fractions run on an SDS-8% (w/v) PAGE, transferred onto nitrocellulose and blotted with a streptavidin-HRP conjugate. A major band running at 90 kDa (p90) was detected. The same procedure was used to quantitatively purify p90 from native cell lysates for N-terminal protein sequencing. The sequence corresponded to the N-terminal sequence of the transferrin receptor (TfR) (Schneider *et al.*, 1983). The identity of p90, was confirmed by analyzing immunoprecipitates of SKBR3 lysates obtained with H7 scFv using a monoclonal anti-TfR antibody (White *et al.*, 1992) (Figure 2(d)).

F5 and H7 phage antibodies and native scFv are rapidly internalized by SKBR3 cells

To determine whether F5 and H7 phage antibodies were endocytosed, we incubated phage with SKBR3 cells and identified internalized phage using an anti-pVIII antibody and confocal microscopy (Figure 3). Both F5 and H7 phage gave strong intracellular staining. In contrast, a control anti-botulinum phage antibody (Amersdorfer *et al.*, 1997) gave no intracellular staining and the anti-ErbB2 phage antibody C6.5 gave significantly less intracellular staining. This is consistent with pre-

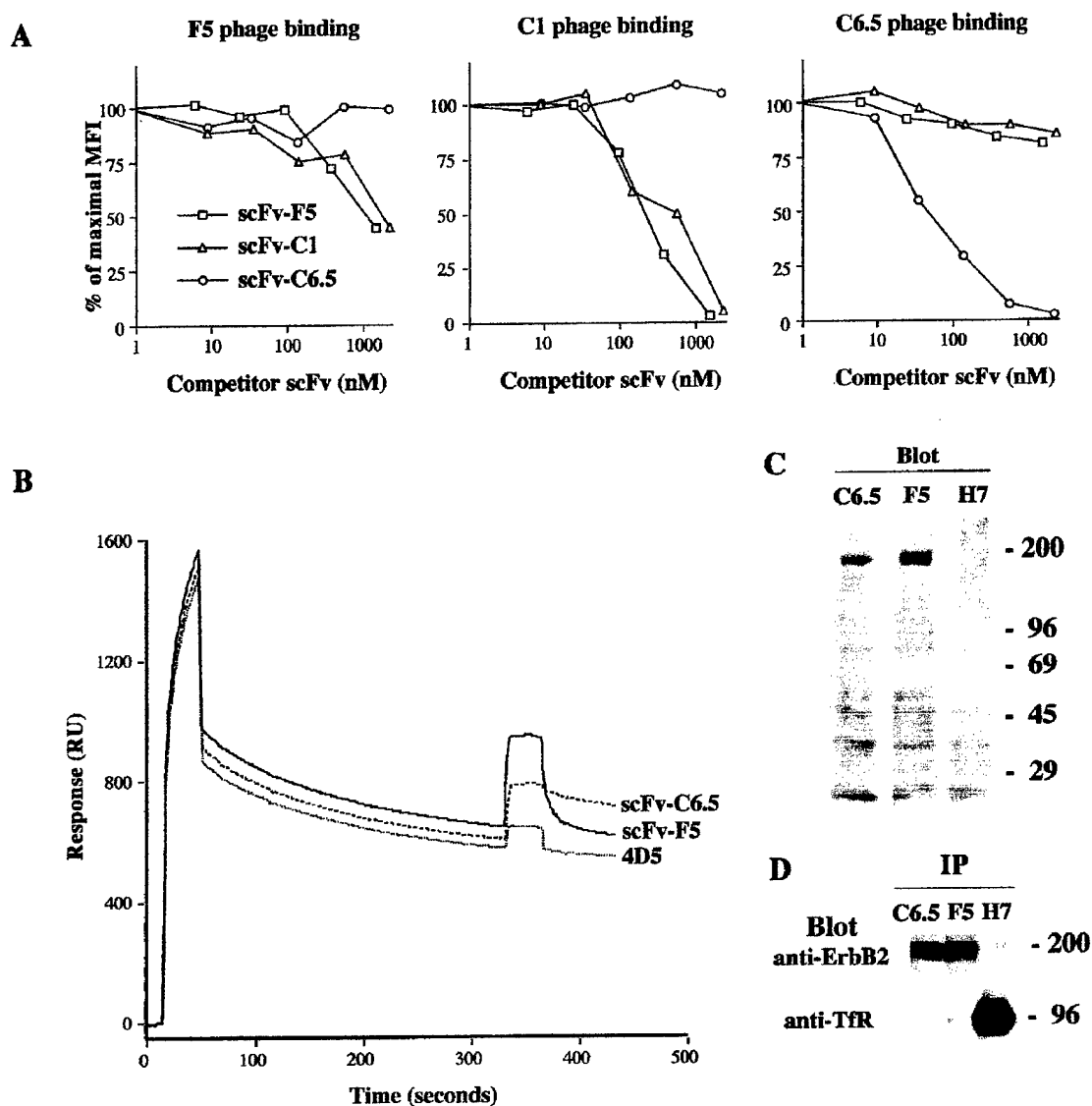


Figure 2. Characterization of anti-ErbB2 and anti-transferrin receptor scFv. (a) Epitope mapping of the F5 and C1 anti-ErbB2 scFv. The epitope recognized by the F5 and C1 scFv selected for internalization were compared to the ErbB2 epitope recognized by the scFv C6.5 selected on recombinant ErbB2 protein. For epitope mapping, the ability of purified C6.5, F5, and C1 scFv to block the binding of F5 phage (left panel), C1 phage (middle panel), and C6.5 phage (right panel) to ErbB2 expressing SKBR3 cells was determined by flow cytometry. F5 and C1 compete with each other for binding and recognize a distinct epitope from C6.5. (b) Epitope mapping of F5 and C6.5 scFv versus 4D5 IgG. The F5 and C6.5 epitopes were compared to the 4D5 epitope by BIAcore. 4D5 IgG was coupled to a sensor chip and ErbB2 was allowed to bind. The ability of C6.5 scFv, F5 scFv and 4D5 IgG to bind to ErbB2 was determined. Both C6.5 and F5 were able to bind, indicating a distinct epitope from 4D5. (c) Western blot of SKBR3 cell lysate using C6.5, F5, and H7 scFv. Both F5 and C6.5 recognize a band the appropriate size for ErbB2. No staining is seen with the H7 scFv. (d) Immunoprecipitation of ErbB2 and transferrin receptor from SKBR3 cell lysate using F5, C6.5 and H7 scFv. After immunoprecipitation with the appropriate scFv, lysates were run on SDS-PAGE, transferred to nitrocellulose and stained with either anti-ErbB2 or anti-transferrin receptor antibody. All scFv were able to immunoprecipitate their target antigen.

vious studies showing minimal endocytosis of C6.5 phage (Becerril *et al.*, 1999). Internalization was detected as soon as five minutes after application of F5-phage and 15 minutes after application of H7-phage (not shown). Purified and gel-filtered

native scFv was also analyzed for internalization into SKBR3 cells by confocal microscopy, with endocytosed scFv detected with the monoclonal antibody 9E10 which recognizes the C-terminal myc-tag. As previously shown, both F5 and H7

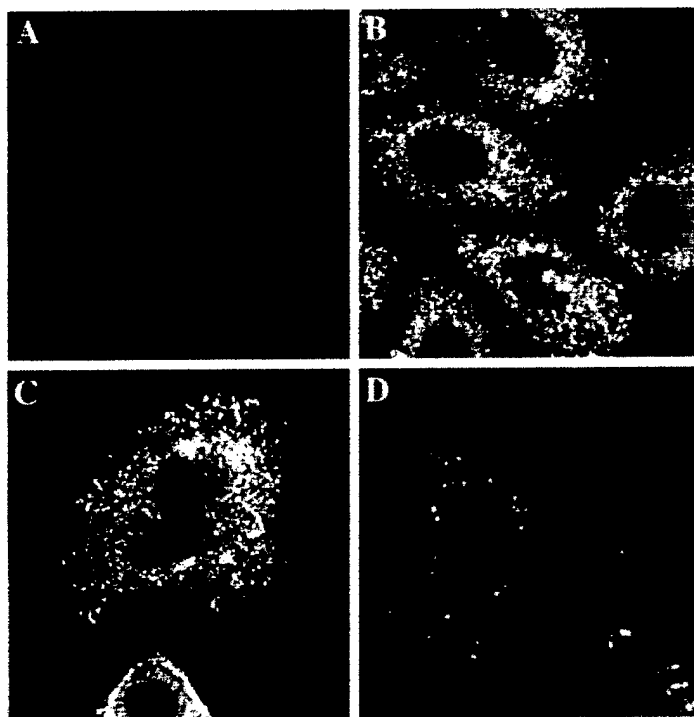


Figure 3. F5 anti-ErbB2 and H7 anti-transferrin phage are endocytosed by SKBR3 cells. Cells were incubated with either anti-ErbB2 phage antibodies F5 (b) and C6.5 (d), anti-transferrin receptor phage antibody H7 (c), or an irrelevant anti-botulinum phage antibody (a). Endocytosis was determined by staining with anti-M13 antibody and analyzing the results by confocal microscopy. Only F5 and H7 phage antibodies show significant intracellular staining.

scFv were monomeric in solution (Figure 1). Both F5 and H7 scFv gave strong intracellular staining, whereas no intracellular staining was seen using the control anti-botulinum scFv and minimal intracellular staining was observed with the anti-ErbB2 C6.5 scFv (Figure 4).

Growth inhibitory effects of F5 and H7 scFv on SKBR3 cells

Since the H7 and F5 antibodies bound to cell surface receptors in a manner that induced endocytosis, we evaluated whether there was any associ-

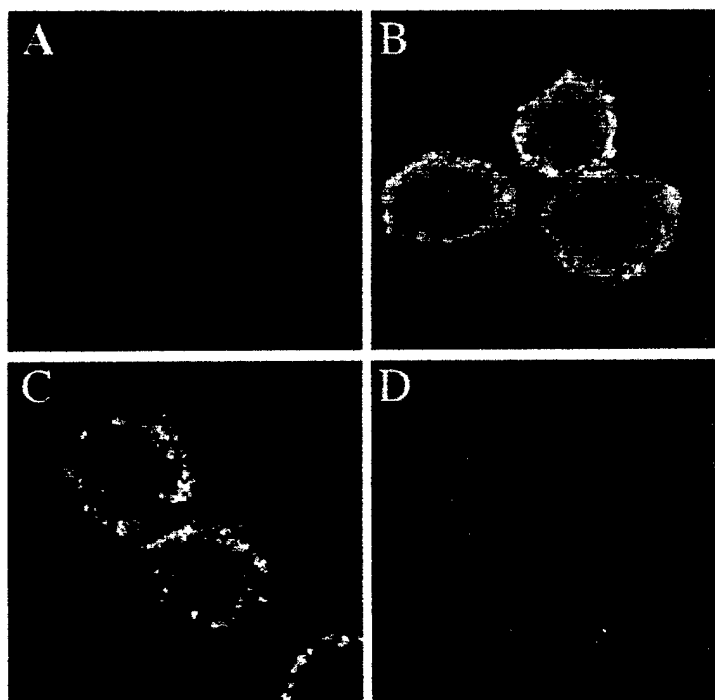


Figure 4. F5 anti-ErbB2 and H7 anti-transferrin receptor scFv are endocytosed by SKBR3 cells. Cells were incubated with either anti-ErbB2 scFv F5 (b) and C6.5 (d), anti-transferrin receptor scFv H7 (c), or an irrelevant anti-botulinum scFv (a). Endocytosis was determined by staining with an anti-myc tag antibody, which recognizes a C-terminal epitope tag on the scFv, and analyzing the results by confocal microscopy. Only F5 and H7 phage scFv show significant intracellular staining.

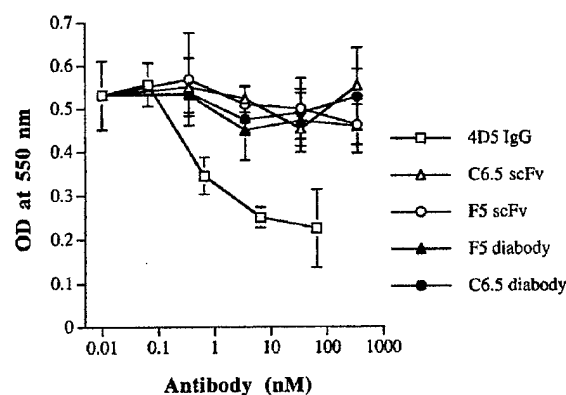


Figure 5. Effects of anti-ErbB2 antibodies F5, C6.5 and 4D5 on SKBR3 cell growth. The ability of F5 scFv, F5 diabody, C6.5 scFv, C6.5 diabody, and 4D5 IgG to inhibit the growth of SKBR3 cells was determined. Only 4D5 showed a dose-dependent growth inhibition.

ated biologic activity with respect to growth inhibition. As antibody-induced internalization can potentially increase the turnover of ErbB2 receptors, reduce the density of cell surface receptors and have an effect on cell growth (Sarup *et al.*, 1991; Tagliabue *et al.*, 1991), we tested the effects of F5 scFv on SKBR3 cell growth. F5 scFv had no effect on cell growth at concentrations up to 300 nM (10 μ g/ml) while the control mAb 4D5 inhibited cell growth of 50% after five days of culture at a concentration of 5 nM as published (Sarup *et al.*, 1991) (Figure 5). Since no inhibitory effect had been observed with monovalent derivatives of growth inhibitory ErbB2 antibodies (Sarup *et al.*, 1991; Shawver *et al.*, 1994), we constructed a bivalent format of F5 scFv (diabody F5) by shortening the linker between the immunoglobulin heavy chain variable domain (V_H) and light chain variable domain (V_L) from 15 to five amino acid residues. This prevents intramolecular pairing of the V_H and V_L resulting in intermolecular pairing and

creation of an scFv dimer termed a diabody (Holliger *et al.*, 1993). The expected size of the F5 diabody was confirmed by gel filtration (Figure 1) and the functional affinity measured as 16 nM. The diabody F5 had no effect on SKBR3 growth (Figure 5). Similarly, neither scFv C6.5 or diabody C6.5 inhibited SKBR3 growth. In contrast the anti-ErbB2 antibody 4D5 showed dose-dependent growth inhibition as an IgG. While the results argue against a growth inhibitory effect for the F5 antibody, the distances between the antigen combining sites as well as binding site flexibility are different for diabodies and IgG. Whether F5 would cause growth inhibition as an IgG is unknown.

We also tested the ability of F5 scFv to induce downstream signaling upon ErbB2 binding. Starved CHO-ErbB2 cells were stimulated with monovalent (scFv) and bivalent (diabody) formats of F5. Both induced weak tyrosine phosphorylation of ErbB2 while the monoclonal antibody 4D5 induced strong phosphorylation (data not shown). The bivalent F5 diabody was also able weakly to activate the MAP kinase cascade as shown by SDS-PAGE band shift using an anti-Erk antibody (data not shown).

H7-scFv was also tested for SKBR3 growth inhibition in parallel with an irrelevant anti-botulinum scFv or with the 4D5 anti-ErbB2 mAb. We observed a strong inhibitory effect (50%) on cell growth using H7-scFv at a concentration of 300 nM (10 μ g/ml). The extent of inhibition obtained was comparable to the maximal effect obtained using 4D5 and no inhibition was obtained with the irrelevant scFv (Figure 6(a)). Anti-TfR antibodies generated using hybridoma technology have also been associated with a growth inhibitory effect (Kovar *et al.*, 1995; Valentini *et al.*, 1994). To investigate the mechanism of the H7 scFv antagonist effect on cell growth, we studied the effect of holotransferrin (iron charged transferrin) on the binding of H7-phage antibodies to SKBR3 cells. Holotransferrin was able to inhibit H7 phage antibody binding to SKBR3 cells (IC_{50} 10 nM) (Figure 6(b)). Control experiments included inhibition of H7-phage binding with soluble scFv-H7

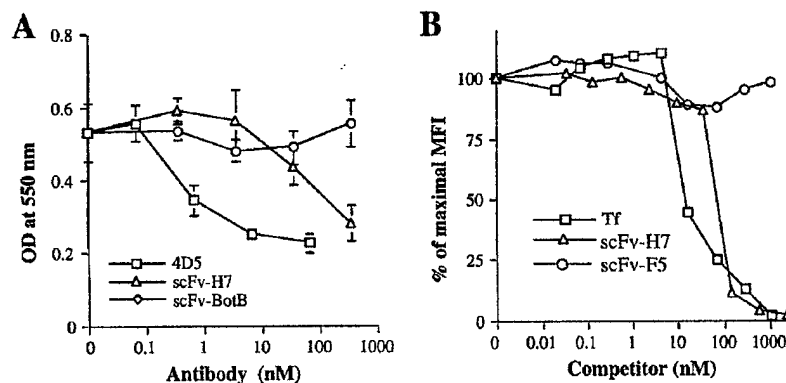


Figure 6. The anti-transferrin scFv H7 inhibits the growth of SKBR3 cells and is a mimic of the ligand holotransferrin. (a) Comparison of the growth inhibitory effect of anti-transferrin receptor H7 scFv and anti-ErbB2 IgG 4D5 on SKBR3 cells. Both antibodies exhibited dose-dependent growth inhibition. (b) Competition between H7 scFv and holotransferrin for binding to SKBR3 cells.

(IC₅₀ 100 nM) and non-inhibition by irrelevant anti-ErbB2 F5 scFv. Holotransferrin also did not inhibit binding of SKBR3 cells by anti-ErbB2 F5-phage antibody. We conclude that the H7 scFv is an antagonist of transferrin binding to TfR. Transferrin, the physiological ligand of TfR, is a major carrier for iron and is rapidly internalized upon TfR binding. H7 scFv's inhibitory effect on SKBR3 growth may result from the combined effects of inhibition of holotransferrin endocytosis and of down regulation of TfR from the cell surface leading to intracellular iron depletion.

Comparison of internalization of F5 phage versus C6.5 phage

We have previously shown that C6.5 scFv displayed monovalently in a phagemid system was only minimally internalized, either as analyzed by confocal microscopy or by recovery of infectious phage from within the cytosol. In this system, enrichment ratios for C6.5 phagemids were only sevenfold above background, suggesting that successful selection from a library of monoclonal binders would be difficult. To understand better the successful selection of F5 and other monovalently displayed scFv from a phagemid library, we compared the internalization rate of F5 phagemid versus C6.5 phage with respect to recovery of infectious phage particles. After 120 minutes of incubation with 3.0×10^3 to 3.0×10^9 phage, significantly more F5 phagemid were recovered than C6.5 phagemid. In fact, F5 scFv displayed monovalently in a phagemid was taken up by ErbB2

expressing cells to a comparable extent as C6.5 scFv displayed multivalently in a phage vector (Figure 7).

Discussion

Phage antibody libraries have become an important resource for the development of reagent, diagnostic, and therapeutic antibodies. Large non-immune libraries serve as a single pot resource for the rapid generation of human antibodies to a wide range of self and non-self antigens, including tumor growth factor receptors. Most of the antibodies isolated from combinatorial libraries expressed on phage have been selected using purified antigens or peptides immobilized on artificial surfaces. This approach may select antibodies that do not recognize the native protein in a physiologic context, especially with large molecular mass cell surface receptors. Attempts have been made to select antigen in native conformation using either cell lysates (Parren *et al.*, 1996; Sanna *et al.*, 1995; Sawyer *et al.*, 1997) fixed cells (Van Ewijk *et al.*, 1997) or living cells (Andersen *et al.*, 1996; Cai & Garen, 1995; de Kruif *et al.*, 1995; Marks *et al.*, 1993; Osbourn *et al.*, 1998; Siegel *et al.*, 1997). Such approaches, because of the heterogeneity of the starting material, require elaborate protocols including subtractive steps to avoid the selection of irrelevant antibodies. The few successful selections performed on heterogenous material were generally done using small libraries from immunized sources. There are only three reports of successful selection on cells using large non-immune libraries (de Kruif *et al.*, 1995; Marks *et al.*, 1993; Vaughan *et al.*, 1996). The use of immunized libraries limits the spectrum of antigen specificities that can potentially be obtained from the same library and typically yield murine antibodies.

The step limiting the selection of binders from large naïve libraries by cell panning seems to be the relatively high background binding of non-specific phage and relatively low binding of specific phage (Becerril *et al.*, 1999; Pereira *et al.*, 1997; Watters *et al.*, 1997). The low binding of specific phage is partially related to the low concentration of a given binding phage in the polyclonal preparation (approximately 1.6×10^{-17} M for a single member of a 10^9 library in a phage preparation of 1.0×10^{13} particles/ml). The low concentration simultaneously limits the efficiency of both subtraction of common binders and enrichment of specific binders. To overcome this limitation, we sought to take advantage of normal cell surface receptor biology. Many receptors undergo endocytosis upon ligand binding. Antibodies can mimic this process, causing receptor aggregation and endocytosis of the antibody upon binding. We hypothesized that enrichment ratios of specific binders could be significantly increased by recovering endocytosed phage antibodies from the cytosol after stringent removal of non-specific phage from

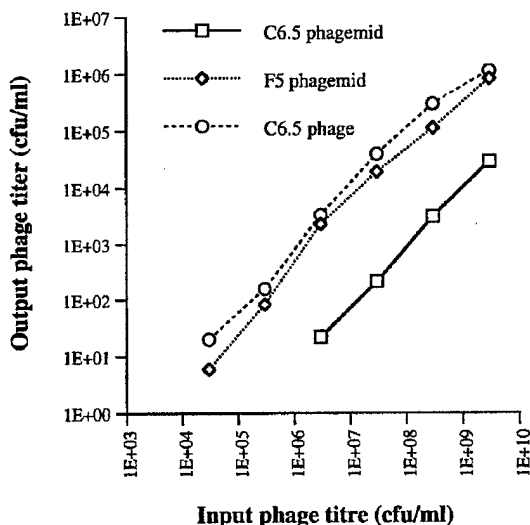


Figure 7. Titer of endocytosed phage as a function of applied phage titer. Varying concentrations of F5 phagemid, C6.5 phagemid, or C6.5 phage (input) were incubated with SKBR3 cells. Surface bound phage were removed with multiple low pH glycine washes and the titer on internalized phage (output) measured by infection of *E. coli*.

the cell surface. Using a model system employing an anti-ErbB2 phage antibody, we found that enrichment of specific *versus* non-specific phage ranged from 3.5 to 146-fold for endocytosed phage compared to 2.7 to 20-fold for surface-bound phage (Becerril *et al.*, 1999). However, the highest values were found only for dimeric antibody species, either dimeric diabodies displayed monovalently in a phagemid vector or scFv displayed multivalently in a phage vector. This is not surprising, since the literature indicates that with rare exceptions, antibodies must be bivalent IgG to induce receptor cross-linking and endocytosis. All large non-immune libraries display monovalent antibody fragments (either scFv or Fab) as single copies using a phagemid vector. Thus, successful selection of internalizing antibodies from such libraries would either require that: (1) the scFv formed spontaneous diabody dimers, as has been reported for some scFv; (2) the monovalent scFv mimicked the natural receptor ligand leading to receptor aggregation and endocytosis; or (3) increased phage display levels of some scFv resulted in greater than one scFv per phage.

Here, we report the successful application of this methodology to select internalizing antibody fragments from scFv libraries displayed monovalently on phage. A large panel of scFv were selected by panning on the tumor cell line SKBR3 which does not recognize normal human fibroblasts. The relatively small number of scFv analyzed have differing patterns of reactivity for other tumor cell lines. Based on the diversity of binders observed in the small sample analyzed (ten different antibodies out of 18 analyzed), hundreds to thousands of different binders with different specificities are likely to be present. To understand better the properties of the selected antibodies, we studied three in detail, two anti-ErbB2 and one that was determined to bind the transferrin receptor. All three were efficiently endocytosed into the target cell line, both as phage and as native monomeric scFv antibody fragments. Somewhat to our surprise, the three scFv did not spontaneously dimerize or aggregate as an explanation for their efficient endocytosis. Rather, the data suggest that some scFv, such as the anti-transferrin receptor antibody, act as ligand mimetics resulting in conformational receptor changes which trigger endocytosis. This may also be the case with the anti-ErbB2 scFv; however, this cannot be studied, since the natural ligand for ErbB2 homodimerization is unknown. Since the anti-ErbB2 scFv recognize ErbB2 in a Western blot, it is unlikely that they are endocytosed by binding an epitope present only on dimerized ErbB2.

When compared to the model C6.5 anti-ErbB2 scFv, the internalizing anti-ErbB2 F5 scFv was endocytosed as efficiently when displayed monomerically in a phagemid system as C6.5 displayed multivalently on phage. This result explains how we were able successfully to select internalizing antibodies from an scFv phagemid library and reconciles our results with observations from the

model system. Our results indicate that selection of antibody fragment libraries displayed on phagemid yields antibodies which are endocytosed as monomers. This is likely to be only a small subset of antibodies capable of triggering receptor-mediated endocytosis, limited to those antibodies capable of mimicking natural ligand binding or otherwise inducing conformational receptor changes leading to receptor aggregation. Most antibodies require a multivalent format to induce receptor cross-linking and endocytosis. Thus, construction of diabody libraries in a phagemid vector or scFv or Fab libraries in a phage vector (Griffiths *et al.*, 1994) should open up this selection approach to more epitopes on more target antigens. Our model system results indicate that the most efficient selection format would be display on phage, an approach which is presently under investigation.

The major advantage of selecting for internalizing antibodies is that one selects for antibodies that trigger a biologic function, not just antibodies that bind. In this case the biologic function is receptor-mediated endocytosis. Such antibodies are likely to have significant therapeutic utility. Use of receptor-mediated endocytosis as a drug delivery route allows delivery of the therapeutic agent specifically into target cells that overexpress the receptor, thereby increasing efficacy while reducing systemic toxicity. In addition, many "drugs" require delivery into the cell for activity (for example, genes and toxins). In some instances, internalization can also be used as a surrogate marker for desirable biological effects of the antibody, for example apoptosis, growth inhibition or growth stimulation. Indeed, we observed a significant growth inhibitory effect of the anti-transferrin scFv on cancer cells. Thus, antibodies selected using this approach may have a direct therapeutic effect, as well as the ability to deliver drugs into the cytosol. Since many antibodies generated by conventional means are not endocytosed, this selection strategy provides a more efficient route to generating internalizing antibodies compared to selecting on protein antigens and screening antibodies for endocytosis. For example, screening the same non-immune library on recombinant ErbB2 extracellular domain did not yield either the F5 or C1 internalizing scFv, perhaps because their K_D values were significantly higher than other anti-ErbB2 scFv isolated.

As an indicator of potential therapeutic utility of antibodies selected for internalization, we have conjugated the F5 anti-ErbB2 scFv to the surface of commercial liposomal doxorubicin converting it into fully functional doxorubicin-loaded anti-ErbB2 immunoliposomes (Nielsen *et al.*, unpublished results). The resulting immunoliposomes have superior efficacy in ErbB2 overexpressing mouse xenograft models compared to untargeted liposomal doxorubicin. Based on preclinical data, expression of the F5 scFv has been scaled up for toxicology studies, cGMP manufacture, and an anticipated phase 1 clinical trial in breast cancer commencing in 2001 (Glaser, 1998).

In summary, we have developed a method for selecting internalizing antibody fragments from phage antibody libraries. The approach can be used to generate internalizing antibodies to known receptors and to identify novel cell surface receptors. The antibodies generated can be used to target therapeutic molecules to the cytosol and in some instances will exert a direct cellular biologic effect *via* their ability to modulate receptor function.

Materials and Methods

Cell culture

Normal human fibroblasts and MCF7 cells were grown in DMEM, 10% (v/v) fetal bovine serum (FBS) (Hyclone), normal human breast cell line Hs 518Bst (ATCC) in DMEM, 10% fetal calf serum (FCS) complemented with 10 µg/ml bovine insulin and 30 ng/ml epidermal growth factor (EGF), SKBR3 in RPMI, 10% FBS, CHO in F12, 10% FBS and CHO-EGFR (Morrison *et al.*, 1993) and CHO-ErbB2 (a gift from Keith Marshall) in F12, 10% complemented with 0.5 mg/ml G418.

Selection of internalizing phage antibodies

A total of five million freshly trypsinized normal human fibroblasts and 10^{12} cfu of the phage library (Sheets *et al.*, 1998) were diluted in 10 ml of ice-cold RPMI, 10% FCS and added to sub-confluent SKBR3 cells grown in a 10 cm diameter plate. After 1.5 hours of incubation at 4°C on a rocker, the cells were washed six times with PBS, covered with prewarmed culture medium and returned to 37°C. After 15 minutes, the cell surface was stripped by three incubations of ten minutes with 4 ml of glycine buffer (500 mM NaCl, 0.1 M glycine (pH 2.5)). The cells were then trypsinized washed with 50 ml of PBS again, lysed with 1 ml of 100 mM TEA for four minutes at 4°C and neutralized with 0.5 ml of 0.5 M Tris (pH 7.4). The phage content of the TEA lysate and the first two glycine washes (neutralized with 1 ml of 0.5 M Tris (pH 7.4)) was titered by infection of *Escherichia coli* TG1 to monitor the selection. Internalized phage (TEA lysate) were amplified for another round of selection. Three rounds of selection were performed.

Initial characterization of binders by ELISA

After two and three rounds of selection, soluble scFv was expressed (De Bellis & Schwartz, 1990) from single colonies grown in 96-well microtiter plates as described (Marks *et al.*, 1991). Crude culture supernatant were tested in ELISA for ErbB2 binding as described (Schier *et al.*, 1996a). In parallel, the bacterial supernatant was tested by cell ELISA on SKBR3 cells and on fibroblasts. Cells were distributed in conical 96-well plates (500,000 cells per well) and then centrifuged at 300 g for three minutes. The cell pellet was resuspended in 100 µl of bacterial supernatant diluted twofold with PBS, 4% (v/v) skimmed milk and incubated for one hour at 4°C on a rocker. After two washes with cold PBS (done by resuspending the cell pellet in 180 µl of PBS and a three minute centrifugation at 300 g), the bound scFv were detected *via* their C-terminal myc-tag (Munro & Pelham, 1986) using the monoclonal antibody 9E10 and peroxidase conjugated anti-mouse Fc (Sigma). The diversity of

ELISA positive clones was determined by PCR amplifying and DNA fingerprinting the scFv gene with *Bst*NI as described (Marks *et al.*, 1991). Unique scFv fingerprint patterns were sequenced using a Sequitherm sequencing kit (Epicentre).

ScFv expression and purification

To facilitate purification of soluble scFv, the scFv genes were subcloned into the expression vector pUC119mycHis (Schier *et al.*, 1995) resulting in the addition of a hexahistidine tag at the C-terminal end of the scFv. The scFv were purified from periplasmic fractions of *E. coli* TG1 by IMAC (Hochuli *et al.*, 1988), using a Ni-NTA column (Qiagen), and gel filtration, as published (Schier *et al.*, 1996b) except that the running buffer after gel filtration was PBS instead of hepes-buffered saline (HBS) for cell culture applications. Alternatively, the scFv genes were PCR amplified using the primer LMB3 (Marks *et al.*, 1991) and fd-FLAG primers before subcloning into pUC119mycHis, resulting in the addition of the flag tag at the N terminus of the scFv.

Immunofluorescence

Cells were grown on coverslips to 50% of confluency in six well-plates and incubated with phage particles or purified scFv for two hours at 37°C. The coverslips were washed six times with PBS, three times for ten minutes with glycine buffer (50 mM glycine (pH 2.8), 500 mM NaCl), neutralized with PBS and fixed with PBS containing 4% (w/v) paraformaldehyde for five minutes at RT. Cells were permeabilized with cold acetone for 30 seconds, and saturated with PBS, 1% BSA. Antibodies were diluted with saturation solution. Intracellular phages were detected with biotinylated anti-M13 polyclonal antibody directed against the pVIII major phage coat protein (5 Prime, 3 Prime Inc.) and streptavidin-Texas Red conjugate (Amersham) both diluted 300 times. Intracellular scFv were detected using the 9E10 mAb (1 µg/ml) (Santa Cruz), an anti-mouse biotinylated antibody (Amersham; diluted 200 times) and streptavidin-Texas Red. Coverslips were inverted on a slide on mounting medium and optical confocal sections were taken using a Bio-Rad MRC 1024 scanning laser confocal microscope. Immunofluorescent microscopy was performed with a Zeiss Axioskop UV fluorescent microscope.

Analysis of phage binding by flow cytometry

Experiments were performed at 4°C. Aliquots of 100,000 cells resuspended in FACS buffer (PBS, 1% FBS) were distributed in conical 96-well microtiter plates and incubated with 100 µl of phage antibodies (typically titrating about 5.0×10^{12} cfu/ml) diluted in PBS, 4% milk for one hour at 4°C. After two PBS washes, bound phage were detected by resuspending the cell pellet in 100 ml of biotinylated anti-M13 sheep antibody diluted 300 times in FACS buffer (30 minutes). Cells were washed again and incubated with streptavidin-phycoerythrin conjugated (PE) (Jackson) for 15 minutes and analyzed using a FacScan (Becton Dickinson). For competition experiments, SKBR3 cells were preincubated with various concentrations of soluble scFv or holo-transferrin (Sigma) for one hour at 4°C. Phage antibodies were added (titer between 10^9 and 5.0×10^9 cfu per

well), incubated one hour at 4°C and bound phage detected as described above.

Affinity measurement and epitope mapping with the BIAcore

On and off-rates were determined using SPR in a BIAcore1000. Approximately 800 RU of ErbB2 ECD were coupled to a CM5 sensor chip as described (Schier, 1995). Association and dissociation rates were measured under continuous flow of HBS at 15 µl/minute using concentrations ranging from 100 nM to 1200 nM and calculated using the BIAanalysis software. For epitope mapping, mAb 4D5 was diluted to 10 µg/ml in 10 mM sodium acetate (pH 4.5), for direct immobilization of 3000 RU to the chip surface.

Affinity measurement on cells

SKOV3 cells were grown to 80-90% confluence in RPMI supplemented with 10% FCS. Cells were harvested by trypsinization. ScFv were incubated with 1×10^5 cells for two hours at varying concentrations. Cell binding was at room temperature in PBS containing 1% (w/v) BSA and 0.1% (w/v) sodium azide in a total volume of 200 µl. After two washes in PBS/BSA, bound scFv was detected with saturating amounts of FITC-labeled anti-FLAG clone M1 (10 µg/ml). After 30 minutes of incubation cells were washed twice and resuspended in PBS containing 1% paraformaldehyde. Fluorescence was measured in a FACSort™ and median fluorescence calculated using the Cellquest™ software and K_D calculated (Benedict *et al.*, 1997).

Cell growth inhibition assay

A total of 100 µl of 10^5 cells/ml were plated in 96-well plates. Four hours later, 100 µl of antibody solutions diluted in culture medium were added and cells incubated for three to five days. The number of living cells was estimated using the CellTiter 96 AQueous cell growth assay kit (Promega).

Western blot and immunoprecipitation using scFv antibodies

SKBR3 cell extracts were prepared using 0.5 ml of lysis buffer (0.4% (v/v) NP40, 50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM DTT, 1 mM PMSF, aprotinin, leupeptin) per confluent 10 cm plate. Cell lysates were run on a SDS-PAGE and transferred onto nitrocellulose membranes. Blots were incubated with scFv (10 µg/ml in PBS, 0.05%, Tween 1% BSA) overnight at 4°C. Blots were washed and scFv detected using 9E10 anti-myc tag antibody (0.1 µg/ml) and HRP conjugated anti-mouse Ig (Amersham). For immunoprecipitation, a dialyzed periplasmic fraction containing the scFv from a 500 ml culture of *E. coli* TG1 was loaded onto 500 µl of a Ni-NTA agarose column. The beads were washed once with PBS, 35 mM imidazole. Then 100 µl of the scFv-loaded Ni-NTA agarose (50% slurry) was used to immunoprecipitate 0.5 ml of SKBR3 cell extract. Immunoprecipitates were analyzed by Western blotting using scFv-F5 or scFv-H7, anti-ErbB2 (Santa Cruz) or anti-human transferin receptor (TfR) H68.4 mAb (White *et al.*, 1992) (a gift from Keith Mostov, UCSF). Alternatively, the cell surface was biotinylated prior to cell lysis and immunoprecipitation. Cells (from a 10 cm diameter confluent plate) were

washed twice with cold PBS and incubated with 3 ml of a 0.1 mg/ml solution of Sulfo-NHS-LC-biotin (Pierce) freshly dissolved in PBS containing 0.1 mM CaCl_2 and 1 mM MgCl_2 at 4°C for 20 minutes. The reaction was quenched by two washes with cold PBS, 50 mM glycine. After a final wash with PBS, cells were lysed with 0.5 ml of lysis buffer. Immunoprecipitation was performed as described above and analyzed by Western blot using HRP-conjugated streptavidin.

Purification of antigen using scFv antibodies

A total of 200 µl of a scFv-H7-Ni-NTA agarose column were loaded twice with a SKBR3 cell lysate corresponding to 3.0×10^6 cells. The column was washed with PBS, 35 mM imidazole, and resuspended directly in 100 µl of Laemli loading buffer 4 ×. The immunoprecipitate was run on a 6% gel, transferred onto PVDF membrane and stained with Ponceau S. The N-terminal protein sequence was determined by Edman sequencing.

Signaling studies

Confluent CHO-ErbB2 cells grown in 6 cm diameter plates were serum starved overnight and stimulated with antibodies for five minutes or one hour and lysed in 300 µl of lysis buffer complemented with sodium orthovanadate. The ErbB2 phosphorylation level was analyzed by Western blot using the anti-phosphotyrosine mAb 4G10 (UBI) and HRP-conjugated anti-mouse IgG (Amersham). ErbB2 levels were checked with the anti-ErbB2 C-18 rabbit polyclonal antibodies (Santa Cruz). MAPkinases Erk1 and Erk2 were detected using the anti-Erk1 K-23 antibody (Santa Cruz) that cross-reacts with Erk2.

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